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(71) Applicant: IMMUNEX CORPORATION [US/US]; Law Dept., 51 University Street, Seattle, WA 98101 (US).

(72) Inventor: DEGLI-ESPOSTI, Mariapia; 5 Aries Court, Shelley, Perth, W.A. 6148 (AU).

(74) Agent: ANDERSON, Kathryn, A.; Immunex Corporation, Law Dept., 51 University Street, Seattle, WA 98101 (US).

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(54) Title: TRAIL RECEPTOR

(57) Abstract

A protein designated TRAIL receptor binds the protein known as TNF-Related Apoptosis-Inducing Ligand (TRAIL). The TRAIL receptor finds use in purifying TRAIL or inhibiting activities thereof. Isolated DNA sequences encoding TRAIL-R polypeptides are provided, along with expression vectors containing the DNA sequences, and host cells transformed with such recombinant expression vectors. Antibodies that are immunoreactive with TRAIL-R are also provided.

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TITLE
TRAIL RECEPTOR

BACKGROUND OF THE INVENTION

A protein known as TNF-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family of ligands (Wiley et al., *Immunity*, 3:673-682, 1995). TRAIL has demonstrated the ability to induce apoptosis of certain transformed cells, including a number of different types of cancer cells as well as virally infected cells (PCT application WO 97/01633 and Wiley et al., *supra*). Identification of receptor protein(s) that bind TRAIL would prove useful in further study of the biological activities of TRAIL.

SUMMARY OF THE INVENTION

The present invention is directed to a novel protein designated TRAIL receptor (TRAIL-R), which binds to a protein known as TNF-related apoptosis-inducing ligand (TRAIL). DNA encoding TRAIL-R, and expression vectors comprising such DNA, are provided. A method for producing TRAIL-R polypeptides comprises culturing host cells transformed with a recombinant expression vector encoding TRAIL-R, under conditions that promote expression of TRAIL-R, then recovering the expressed TRAIL-R polypeptides from the culture. Antibodies that are immunoreactive with TRAIL-R are also provided.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B present the nucleotide sequence of a human TRAIL receptor cDNA, as well as the amino acid sequence encoded thereby.

Figures 2A and 2B present the nucleotide and encoded amino acid sequences for a second human TRAIL receptor cDNA clone. The amino acid sequence of Figures 2A-2B differs at two positions from the sequence presented in Figures 1A-1B.

DETAILED DESCRIPTION OF THE INVENTION

A novel protein designated TRAIL receptor (TRAIL-R) is provided herein. TRAIL-R binds to the cytokine designated TNF-related apoptosis-inducing ligand (TRAIL). Certain uses of TRAIL-R flow from this ability to bind TRAIL, as discussed further below. TRAIL-R finds use in inhibiting biological activities of TRAIL, or in purifying TRAIL by affinity chromatography, for example.

immunogens to generate antibodies that are immunoreactive therewith. In one embodiment of the invention, the antibodies are monoclonal antibodies.

The nucleotide sequence of a human TRAIL receptor cDNA is presented in Figures 1A to 1B, along with the amino acid sequence encoded by the cDNA (SEQ ID NO:1 and SEQ ID NO:2). Figures 2A to 2B (SEQ ID NO:3 and SEQ ID NO:4) present the nucleotide sequence of a second human TRAIL receptor cDNA clone, and the amino acid sequence encoded thereby. The nucleotide sequences of Figures 1 and 2 differ at two positions. The nucleotide at position 145 is a C in the sequence of Figure 1 (SEQ ID NO:1), whereas nucleotide 145 is a T in Figure 2 (SEQ ID NO:3); the nucleotide at position 971 is a C in Figure 1 (SEQ ID NO:1), but is a T in Figure 2 (SEQ ID NO:3). The amino acid sequences likewise differ at two positions. Residue 35 is Pro in Figure 1 (SEQ ID NO:2) and Ser in Figure 2 (SEQ ID NO:4); residue 310 is Ser in Figure 1 (SEQ ID NO:2) and Leu in Figure 2 (SEQ ID NO:4). One possible explanation is that the TRAIL receptors of Figures 1 and 2 are allelic variants.

The sequence information presented in Figures 1 and 2 identifies the TRAIL receptor protein as a member of the tumor necrosis factor receptor (TNF-R) family of receptors (reviewed in Smith et al., *Cell* 76:959-962, 1994). The TRAIL-R proteins include certain features of other proteins of this family, including cysteine rich repeats in the extracellular domain, as discussed below. However, TRAIL-R lacks a so-called "death domain", which is found in the cytoplasmic region of certain other receptor proteins. Such domains have been reported to be associated with transduction of apoptotic signals, i.e., to play a role in initiating intracellular apoptotic signaling cascades. Cytoplasmic death domains have been identified in Fas antigen (Itoh and Nagata, *J. Biol. Chem.* 268:10932, 1993), TNF receptor type I (Tartaglia et al. *Cell* 74:845, 1993), DR3 (Chinnaiyan et al., *Science* 274:990-992, 1996), and CAR-1 (Brojatsch et al., *Cell* 87:845-855, 1996).

The TRAIL-R proteins of Figure 1 (SEQ ID NO:2) and Figure 2 (SEQ ID NO:4) include an N-terminal hydrophobic region that functions as a signal peptide, followed by an extracellular domain, a transmembrane region comprising amino acids 212 through 232, and a C-terminal cytoplasmic domain comprising amino acids 233 through 386. Computer analysis predicts that the signal peptide is likely to be cleaved after residue 55. Cleavage of the signal peptide thus would yield a mature protein comprising amino acids 56 to 386.

The calculated molecular weight for a mature protein having the amino acid sequence of residues 56 to 386 of Figure 1 is about 36 kilodaltons. The isoelectric point (pI) is predicted to be about 5.27. The skilled artisan will recognize that the molecular weight of particular preparations of TRAIL-R protein may differ, according to such

factors as the degree of glycosylation. The glycosylation pattern of a particular preparation of TRAIL-R may vary according to the type of cells in which the protein is expressed, for example, and a given preparation may include multiple differentially glycosylated species of the protein. TRAIL-R proteins with or without associated native-pattern glycosylation are provided herein. Expression of TRAIL-R in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules. Further, N-glycosylation sites in the native protein may be inactivated, as discussed below.

The present invention encompasses TRAIL-R in various forms, including those that are naturally occurring or produced through various techniques such as procedures involving recombinant DNA technology. Such forms of TRAIL-R include, but are not limited to, fragments, derivatives, variants, and oligomers of TRAIL-R, as well as fusion proteins containing TRAIL-R or fragments thereof.

TRAIL-R may be modified to create derivatives thereof by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of TRAIL-R may be prepared by linking the chemical moieties to functional groups on TRAIL-R amino acid side chains or at the N-terminus or C-terminus of a TRAIL-R polypeptide. Conjugates comprising diagnostic (detectable) or therapeutic agents attached to TRAIL-R are contemplated herein, as discussed in more detail below.

Other derivatives of TRAIL-R within the scope of this invention include covalent or aggregative conjugates of TRAIL-R polypeptides with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. Examples of fusion proteins are discussed below in connection with TRAIL-R oligomers. Further, TRAIL-R-containing fusion proteins can comprise peptides added to facilitate purification and identification of TRAIL-R. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (SEQ ID NO:5), which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the Flag® peptide in the presence of certain divalent metal cations, as described in U.S. Patent 5,011,912, hereby incorporated by reference. The 4E11 hybridoma cell line has been deposited with the American Type Culture Collection under accession no. HB 9259. Monoclonal antibodies that bind the Flag® peptide are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut.

Both cell membrane-bound and soluble (secreted) forms of TRAIL-R are provided herein. Soluble TRAIL-R may be identified (and distinguished from non-soluble

membrane-bound counterparts) by separating intact cells expressing a TRAIL-R polypeptide from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The presence of TRAIL-R in the medium indicates that the protein was secreted from the cells and thus is a soluble form of the desired protein.

Soluble forms of receptor proteins typically lack the transmembrane region that would cause retention of the protein on the cell surface. In one embodiment of the invention, a soluble TRAIL-R polypeptide comprises the extracellular domain of the protein. In certain other embodiments, soluble TRAIL-R polypeptides are fragments of the extracellular domain. A soluble TRAIL-R polypeptide may include the cytoplasmic domain, or a portion thereof, as long as the polypeptide is secreted from the cell in which it is produced.

Examples of soluble TRAIL-R include, but are not limited to, polypeptides comprising amino acids 56 through 211 of Figure 1 or 2 (the extracellular domain) or amino acids 56 to 208 of Figure 1 or 2 (a fragment of the extracellular domain). An expression vector encoding a soluble TRAIL-R polypeptide comprising amino acids 1 through 208 of Figures 1 or 2, fused to an antibody-derived Fc polypeptide, is described in example 3 below. Further examples include, but are not limited to, fragments of the extracellular domain that include the cysteine-rich repeats of TRAIL-R, as described below.

Soluble forms of TRAIL-R possess certain advantages over the membrane-bound form of the protein. Purification of the protein from recombinant host cells is facilitated, since the soluble proteins are secreted from the cells. Further, soluble proteins are generally more suitable for certain applications, e.g., for intravenous administration.

TRAIL-R fragments are provided herein. Such fragments may be prepared by any of a number of conventional techniques. Desired peptide fragments may be chemically synthesized. An alternative involves generating TRAIL-R fragments by enzymatic digestion, e.g., by treating the protein with an enzyme known to cleave proteins at sites defined by particular amino acid residues. A TRAIL-R DNA may be digested with suitable restriction enzymes, to derive a DNA fragment encoding a desired polypeptide fragment. Yet another suitable technique involves isolating and amplifying a DNA fragment encoding a desired polypeptide fragment, by polymerase chain reaction (PCR). Oligonucleotides that define the desired termini of the DNA fragment are employed as the 5' and 3' primers in the PCR.

TRAIL-R polypeptide fragments may be employed as immunogens, in generating antibodies. Particular embodiments are directed to TRAIL-R polypeptide fragments that retain the ability to bind TRAIL. Such a fragment may be a soluble TRAIL-R polypeptide, as described above.

In particular embodiments, TRAIL-R fragments include the cysteine-rich repeat motifs found in the extracellular domain. The human TRAIL-R proteins of Figures 1 and 2 contain two such cysteine rich repeats, the first including residues 98 through 139, and the second including residues 140 through 181. Receptors of the TNF-R family contain such cysteine-rich repeats in their extracellular domains (Marsters et al., *J. Biol. Chem.* 267:5747-5750, 1992; Smith et al., *Cell* 76:959-962, 1994). These repeats are believed to be important for ligand binding. To illustrate, Marsters et al., *supra*, reported that soluble TNF-R type 1 polypeptides lacking one of the repeats exhibited a ten fold reduction in binding affinity for TNFα and TNFß; deletion of the second repeat resulted in a complete loss of detectable binding of the ligands.

Residues 182 through 211 of Figures 1 and 2 constitute a spacer region. This spacer is the C-terminal portion of the extracellular domain, positioned between the cysteine rich repeats and the transmembrane region. Such spacer regions have been identified in certain other proteins of the TNF-R family, and reportedly are not critical for ligand binding. TRAIL-R fragments lacking the spacer region are provided herein.

Naturally occurring variants of the TRAIL-R protein of Figures 1 and 2 are provided herein. Such variants include, for example, proteins that result from alternate mRNA splicing events or from proteolytic cleavage of the TRAIL-R protein. Alternate splicing of mRNA may, for example, yield a truncated but biologically active TRAIL-R protein, such as a naturally occurring soluble form of the protein. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the TRAIL-R protein (generally from 1-5 terminal amino acids). TRAIL-R proteins in which differences in amino acid sequence are attributable to genetic polymorphism (allelic variation among individuals producing the protein) are also contemplated herein.

The skilled artisan will also recognize that the position(s) at which the signal peptide is cleaved may differ from that predicted by computer program, and may vary according to such factors as the type of host cells employed in expressing a recombinant TRAIL-R polypeptide. A protein preparation may include a mixture of protein molecules having different N-terminal amino acids, resulting from cleavage of the signal peptide at more than one site.

Regarding the discussion herein of various domains of TRAIL-R protein, the skilled artisan will recognize that the above-described boundaries of such regions of the protein are approximate. To illustrate, the boundaries of the transmembrane region (which may be predicted by using computer programs available for that purpose) may differ from those described above. Thus, soluble TRAIL-R polypeptides in which the C-

terminus of the extracellular domain differs from the residue so identified above are contemplated herein.

Other naturally occurring TRAIL-R DNAs and polypeptides include those derived from non-human species. Homologs of the human TRAIL-R of Figures 1 or 2, from other mammalian species, are contemplated herein, for example. Probes based on the human DNA sequence of Figure 1 or 2 may be used to screen cDNA libraries derived from other mammalian species, using cross-species hybridization techniques.

TRAIL-R DNA sequences may vary from the native sequences disclosed herein. Due to the known degeneracy of the genetic code, wherein more than one codon can encode the same amino acid, a DNA sequence can vary from that shown in Figure 1 (SEQ ID NO:1) or Figure 2 (SEQ ID NO:3) and still encode a TRAIL-R protein having the amino acid sequence presented in those Figures (SEQ ID NO:2 OR 4, respectively). Such variant DNA sequences may result from silent mutations (e.g., occurring during PCR amplification), or may be the product of deliberate mutagenesis of a native sequence. Thus, among the DNA sequences provided herein are native TRAIL-R sequences (e.g., cDNA comprising the nucleotide sequence presented in Figures 1 or 2) and DNA that is degenerate as a result of the genetic code to a native TRAIL-R DNA sequence.

Among the TRAIL-R polypeptides provided herein are variants of native TRAIL-R polypeptides that retain a biological activity of a native TRAIL-R. Such variants include polypeptides that are substantially homologous to native TRAIL-R, but which have an amino acid sequence different from that of a native TRAIL-R because of one or more deletions, insertions or substitutions. Particular embodiments include, but are not limited to, TRAIL-R polypeptides that comprise from one to ten deletions, insertions or substitutions of amino acid residues, when compared to a native TRAIL-R sequence. The TRAIL-R-encoding DNAs of the present invention include variants that differ from a native TRAIL-R DNA sequence because of one or more deletions, insertions or substitutions, but that encode a biologically active TRAIL-R polypeptide. One biological activity of TRAIL-R is the ability to bind TRAIL.

Nucleic acid molecules capable of hybridizing to the DNA of Figures 1 or 2 under moderately stringent or highly stringent conditions, and which encode a biologically active TRAIL-R, are provided herein. Such hybridizing nucleic acids include, but are not limited to, variant DNA sequences and DNA derived from non-human species, e.g., non-human mammals.

Moderately stringent conditions include conditions described in, for example, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Vol. 1, pp 1.101-104, Cold Spring Harbor Laboratory Press, 1989. Conditions of moderate stringency, as defined by Sambrook et al., include use of a prewashing solution of 5X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of about 55°C, 5 X SSC, overnight.

Highly stringent conditions include higher temperatures of hybridization and washing. One embodiment of the invention is directed to DNA sequences that will hybridize to the DNA of Figures 1 or 2 under highly stringent conditions, wherein said conditions include hybridization at 68°C followed by washing in 0.1X SSC/0.1% SDS at 63-68°C.

Certain DNAs and polypeptides provided herein comprise nucleotide or amino acid sequences, respectively, that are at least 80% identical to a native TRAIL-R sequence. Also contemplated are embodiments in which a TRAIL-R DNA or polypeptide comprises a sequence that is at least 90% identical, at least 95% identical, or at least 98% identical to a native TRAIL-R sequence.

The percent identity may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res. 14*:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. For fragments, the percent identity is calculated by comparing the sequence of the fragment with the corresponding portion of a native TRAIL-R.

In particular embodiments of the invention, a variant TRAIL-R polypeptide differs in amino acid sequence from a native TRAIL-R, but is substantially equivalent to a native TRAIL-R in a biological activity. One example is a variant TRAIL-R that binds TRAIL with essentially the same binding affinity as does a native TRAIL-R. Binding affinity can be measured by conventional procedures, e.g., as described in U.S. Patent no. 5,512,457.

Variant amino acid sequences may comprise conservative substitution(s), meaning that one or more amino acid residues of a native TRAIL-R is replaced by a different residue, but that the conservatively substituted TRAIL-R polypeptide retains a desired biological activity of the native protein (e.g., the ability to bind TRAIL). A given amino acid may be replaced by a residue having similar physiochemical characteristics. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other conservative substitutions, e.g., involving substitutions of entire regions having similar hydrophobicity characteristics, are well known.

In another example of variants, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or

disulfide bridges upon renaturation. Cysteine residues within the above-described cysteine rich repeat domains advantageously remain unaltered in TRAIL-R variants, when retention of TRAIL-binding activity is desired.

Other variants are prepared by modification of adjacent dibasic amino acid residues, to enhance expression in yeast systems in which KEX2 protease activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Human TRAIL-R contains such adjacent basic residue pairs at amino acids 75-76, 233-234, 260-261, 261-262, 328-329, and 329-330 of Figure 1 and 2. Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites.

In still other variants, N-glycosylation sites in a native TRAIL-R are inactivated. N-glycosylation sites can be modified to preclude glycosylation, allowing expression of a more homogeneous, reduced carbohydrate analog in mammalian and yeast expression systems. N-glycosylation sites in eukaryotic polypeptides are characterized by an amino acid triplet Asn-X-Y, wherein X is any amino acid except Pro and Y is Ser or Thr. The TRAIL-R protein of Figure 1 and 2 comprises three such triplets, at amino acids 127-129, 171-173, and 182-184. Appropriate substitutions, additions or deletions to the nucleotide sequence encoding these triplets will result in prevention of attachment of carbohydrate residues to the Asn side chain. Alteration of a single nucleotide, chosen so that Asn is replaced by a different amino acid, for example, is sufficient to inactivate an N-glycosylation site. Known procedures for inactivating N-glycosylation sites in proteins include those described in U.S. Patent 5,071,972 and EP 276,846, hereby incorporated by reference.

TRAIL-R polypeptides, including variants and fragments thereof, can be tested for biological activity in any suitable assay. The ability of a TRAIL-R polypeptide to bind TRAIL can be confirmed in conventional binding assays, examples of which are described below.

Expression Systems

The present invention also provides recombinant cloning and expression vectors containing TRAIL-R DNA, as well as host cell containing the recombinant vectors. Expression vectors comprising TRAIL-R DNA may be used to prepare TRAIL-R polypeptides encoded by the DNA. A method for producing TRAIL-R polypeptides

comprises culturing host cells transformed with a recombinant expression vector encoding TRAIL-R, under conditions that promote expression of TRAIL-R, then recovering the expressed TRAIL-R polypeptides from the culture. The skilled artisan will recognize that the procedure for purifying the expressed TRAIL-R will vary according to such factors as the type of host cells employed, and whether the TRAIL-R is membrane-bound or a soluble form that is secreted from the host cell.

Any suitable expression system may be employed. The vectors include a DNA encoding a TRAIL-R polypeptide, operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the TRAIL-R DNA sequence. Thus, a promoter nucleotide sequence is operably linked to an TRAIL-R DNA sequence if the promoter nucleotide sequence controls the transcription of the TRAIL-R DNA sequence. An origin of replication that confers the ability to replicate in the desired host cells, and a selection gene by which transformants are identified, are generally incorporated into the expression vector.

In addition, a sequence encoding an appropriate signal peptide (native or heterologous) can be incorporated into expression vectors. A DNA sequence for a signal peptide (secretory leader) may be fused in frame to the TRAIL-R sequence so that the TRAIL-R is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells promotes extracellular secretion of the TRAIL-R polypeptide. The signal peptide is cleaved from the TRAIL-R polypeptide upon secretion of TRAIL-R from the cell.

Suitable host cells for expression of TRAIL-R polypeptides include prokaryotes, yeast or higher eukaryotic cells. Mammalian or insect cells are generally preferred for use as host cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual*, Elsevier, New York, (1985). Cell-free translation systems could also be employed to produce TRAIL-R polypeptides using RNAs derived from DNA constructs disclosed herein.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or *Bacilli*. Suitable prokaryotic host cells for transformation include, for example, *E.*

coli, Bacillus subtilis, Salmonella typhimurium, and various other species within the genera *Pseudomonas, Streptomyces,* and *Staphylococcus.* In a prokaryotic host cell, such as *E. coli,* a TRAIL-R polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant TRAIL-R polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. An appropriate promoter and a TRAIL-R DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include β-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature 275*:615, 1978; and Goeddel et al., *Nature 281*:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res. 8*:4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage λ P_L promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9, ATCC 37092) and pPLc28 (resident in *E. coli* RR1, ATCC 53082).

TRAIL-R alternatively may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces,* may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem. 255*:2073, 1980) or other glycolytic enzymes (Hess et al.,

J. Adv. Enzyme Reg. 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phospho-glucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* may be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) into the above-described yeast vectors.

The yeast α-factor leader sequence may be employed to direct secretion of the TRAIL polypeptide. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. *See*, e.g., Kurjan et al., *Cell* 30:933, 1982 and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978. The Hinnen et al. protocol selects for Trp^+ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Yeast host cells transformed by vectors containing an ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems also may be employed to express recombinant TRAIL-R polypeptides. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988). Established cell lines of mammalian origin also may be employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC

CRL 1651) (Gluzman et al., *Cell 23*:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CV1/EBNA cell line (ATCC CRL 10478) derived from the African green monkey kidney cell line CV1 (ATCC CCL 70) as described by McMahan et al. (*EMBO J.* 10: 2821, 1991).

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature 273*:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Expression vectors for use in mammalian host cells can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol. 3*:280, 1983), for example. A useful system for stable high level expression of mammalian cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature 312*:768, 1984 has been deposited as ATCC 39890. Additional mammalian expression vectors are described in EP-A-0367566, and in WO 91/18982. As one alternative, the vector may be derived from a retrovirus.

Regarding signal peptides that may be employed in producing TRAIL-R, the native signal peptide of TRAIL-R may be replaced by a heterologous signal peptide or leader sequence, if desired. The choice of signal peptide or leader may depend on factors such as the type of host cells in which the recombinant TRAIL-R is to be produced. To illustrate, examples of heterologous signal peptides that are functional in mammalian host cells include the signal sequence for interleukin-7 (IL-7) described in United States Patent 4,965,195, the signal sequence for interleukin-2 receptor described in Cosman et al., *Nature* 312:768 (1984); the interleukin-4 receptor signal peptide described in EP 367,566; the type I interleukin-1 receptor signal peptide described in U.S. Patent 4,968,607; and the

type II interleukin-1 receptor signal peptide described in EP 460,846. Another example is a leader peptide derived from cytomegalovirus, as described in WO 97/01633, hereby incorporated by reference.

Purified Protein

TRAIL-R polypeptides of the present invention may be produced by recombinant expression systems as described above, or purified from naturally occurring cells. TRAIL-R may be purified by any of a number of suitable methods, which may employ conventional protein purification techniques. As is known to the skilled artisan, procedures for purifying a given protein are chosen according to such factors as the types of contaminants that are to be removed, which may vary according to the particular cells in which the TRAIL-R is expressed. For recombinant proteins, other considerations include the particular expression systems employed and whether or not the desired protein is secreted into the culture medium.

In one method, cells expressing the protein are disrupted by any of the numerous known techniques, including freeze-thaw cycling, sonication, mechanical disruption, or by use of cell lysing agents. Alternatively, a soluble TRAIL-R may be expressed and secreted from the cell. The subsequent purification process may include affinity chromatography, e.g., employing a chromatography matrix containing TRAIL. The chromatography matrix may instead comprise an antibody that binds TRAIL-R. The TRAIL-R polypeptides can be recovered from an affinity chromatography column using conventional techniques (e.g., elution in a high salt buffer), then dialyzed into a lower salt buffer for use.

One example of a suitable affinity chromatography matrix is a Flag®-TRAIL affi-gel column (10 mg of recombinant protein coupled to 1 ml of affi-gel beads). The Affi-gel support is an N-hydroxysuccinimide ester of a derivatized, crosslinked agarose gel bead (available from Biorad Laboratories, Richmond, CA). As discussed above, the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (SEQ ID NO:5), provides an epitope reversibly bound by specific monoclonal antibodies, which allows rapid assay and facile purification of expressed recombinant protein. Preparation of Flag®-TRAIL fusion proteins (comprising Flag® fused to a soluble TRAIL polypeptide) is further described in PCT application WO 97/01633, hereby incorporated by reference. The Flag®-TRAIL fusion protein is attached to the affi-gel beads by conventional techniques.

In another approach, when an expression system that secretes the recombinant protein is employed, the culture medium first may be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a

support materials commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. In addition, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, (e.g., silica gel having pendant methyl or other aliphatic groups) can be employed. Some or all of the foregoing purification steps, in various combinations, may be employed.

Recombinant protein produced in bacterial culture can be isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange, affinity purification or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

In yeast host cells, TRAIL-R is preferably expressed as a secreted polypeptide, to simplify purification. Recombinant polypeptides secreted from a yeast host cell fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). Urdal et al. describe two sequential, reversed-phase HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column.

The desired degree of purity depends on the intended use of the protein. A relatively high degree of purity is desired when the protein is to be administered *in vivo*, for example. Advantageously, TRAIL-R polypeptides are purified such that no protein bands corresponding to other (non-TRAIL-R) proteins are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Those skilled in the pertinent field will recognize that multiple bands corresponding to TRAIL-R protein may be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. TRAIL-R most preferably is purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-PAGE. The protein band may be visualized by silver staining, Coomassie blue staining, or (if the protein is radiolabeled) by autoradiography.

Oligomeric Forms of TRAIL-R

Encompassed by the present invention are oligomers that contain TRAIL-R polypeptides. TRAIL-R oligomers may be in the form of covalently-linked or non-covalently-linked dimers, trimers, or higher oligomers.

One embodiment of the invention is directed to oligomers comprising multiple TRAIL-R polypeptides joined *via* covalent or non-covalent interactions between peptide moieties fused to the TRAIL-R polypeptides. Such peptides may be peptide linkers (spacers), or peptides that have the property of promoting oligomerization. Leucine zippers and certain polypeptides derived from antibodies are among the peptides that can promote oligomerization of TRAIL-R polypeptides attached thereto, as described in more detail below.

In particular embodiments, the oligomers comprise from two to four TRAIL-R polypeptides. The TRAIL-R moieties of the oligomer may be soluble polypeptides, as described above.

As one alternative, a TRAIL-R oligomer is prepared using polypeptides derived from immunoglobulins. Preparation of fusion proteins comprising certain heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (*PNAS USA* 88:10535, 1991); Byrn et al. (*Nature* 344:677, 1990); Hollenbaugh and Aruffo ("Construction of Immunoglobulin Fusion Proteins", in *Current Protocols in Immunology*, Suppl. 4, pages 10.19.1 - 10.19.11, 1992); Smith et al. (*Cell* 73:1349-1360, 1993); and Fanslow et al. (*J. Immunol.* 149:655-660, 1992).

One embodiment of the present invention is directed to a TRAIL-R dimer comprising two fusion proteins created by fusing TRAIL-R to the Fc region of an antibody. A gene fusion encoding the TRAIL-R/Fc fusion protein is inserted into an appropriate expression vector. TRAIL-R/Fc fusion proteins are expressed in host cells transformed with the recombinant expression vector, and allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between the Fc moieties to yield divalent TRAIL-R.

Provided herein are fusion proteins comprising a TRAIL-R polypeptide fused to an Fc polypeptide derived from an antibody. DNA encoding such fusion proteins, as well as dimers containing two fusion proteins joined *via* disulfide bonds between the Fc moieties thereof, are also provided. The term "Fc polypeptide" as used herein includes native and mutein forms of polypeptides derived from the Fc region of an antibody. Truncated forms of such polypeptides containing the hinge region that promotes dimerization are also included.

One suitable Fc polypeptide, described in PCT application WO 93/10151 (hereby incorporated by reference), is a single chain polypeptide extending from the N-terminal hinge region to the native C-terminus of the Fc region of a human IgG1 antibody. Another useful Fc polypeptide is the Fc mutein described in U.S. Patent 5,457,035 and in Baum et al., (*EMBO J.* 13:3992-4001, 1994). The amino acid sequence of this mutein is identical to that of the native Fc sequence presented in WO 93/10151, except that amino acid 19 has been changed from Leu to Ala, amino acid 20 has been changed from Leu to Glu, and amino acid 22 has been changed from Gly to Ala. The mutein exhibits reduced affinity for Fc receptors.

In other embodiments, TRAIL-R may be substituted for the variable portion of an antibody heavy or light chain. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a TRAIL-R oligomer with as many as four TRAIL-R extracellular regions.

Alternatively, the oligomer is a fusion protein comprising multiple TRAIL-R polypeptides, with or without peptide linkers (spacer peptides). Among the suitable peptide linkers are those described in U.S. Patents 4,751,180 and 4,935,233, which are hereby incorporated by reference. A DNA sequence encoding a desired peptide linker may be inserted between, and in the same reading frame as, the DNA sequences encoding TRAIL-R, using any suitable conventional technique. For example, a chemically synthesized oligonucleotide encoding the linker may be ligated between sequences encoding TRAIL-R. In particular embodiments, a fusion protein comprises from two to four soluble TRAIL-R polypeptides, separated by peptide linkers.

Another method for preparing oligomeric TRAIL-R involves use of a leucine zipper. Leucine zipper domains are peptides that promote oligomerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., *Science* 240:1759, 1988), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives thereof that dimerize or trimerize. Examples of leucine zipper domains suitable for producing soluble oligomeric proteins are described in PCT application WO 94/10308, and the leucine zipper derived from lung surfactant protein D (SPD) described in Hoppe et al. (*FEBS Letters* 344:191, 1994), hereby incorporated by reference. The use of a modified leucine zipper that allows for stable trimerization of a heterologous protein fused thereto is described in Fanslow et al. (*Semin. Immunol.* 6:267-278, 1994). Recombinant fusion proteins comprising a soluble TRAIL-R polypeptide fused to a leucine zipper peptide are expressed in suitable host cells, and the soluble oligomeric TRAIL-R that forms is recovered from the culture supernatant.

Oligomeric TRAIL-R has the property of bivalent, trivalent, etc. binding sites for TRAIL. The above-described fusion proteins comprising Fc moieties (and oligomers

formed therefrom) offer the advantage of facile purification by affinity chromatography over Protein A or Protein G columns. DNA sequences encoding oligomeric TRAIL-R, or encoding fusion proteins useful in preparing TRAIL-R oligomers, are provided herein.

Assays

TRAIL-R proteins (including fragments, variants, oligomers, and other forms of TRAIL-R) may be tested for the ability to bind TRAIL in any suitable assay. To illustrate, TRAIL-R may be labeled with a detectable reagent (e.g., a radionuclide, chromophore, enzyme that catalyzes a colorimetric or fluorometric reaction, and the like). The labeled TRAIL-R is contacted with cells expressing TRAIL. The cells then are washed to remove unbound labeled TRAIL-R, and the presence of cell-bound label is determined by a suitable technique, chosen according to the nature of the label.

One example of a binding assay procedure is as follows. A recombinant expression vector containing TRAIL cDNA is constructed, e.g., as described in PCT application WO 97/01633, hereby incorporated by reference. DNA and amino acid sequence information for human and mouse TRAIL is presented in WO 97/01633. TRAIL comprises an N-terminal cytoplasmic domain, a transmembrane region, and a C-terminal extracellular domain. CV1-EBNA-1 cells in 10 cm^2 dishes are transfected with the recombinant expression vector. CV-1/EBNA-1 cells (ATCC CRL 10478) constitutively express EBV nuclear antigen-1 driven from the CMV immediate-early enhancer/promoter. CV1-EBNA-1 was derived from the African Green Monkey kidney cell line CV-1 (ATCC CCL 70), as described by McMahan et al. (*EMBO J.* 10:2821, 1991).

The transfected cells are cultured for 24 hours, and the cells in each dish then are split into a 24-well plate. After culturing an additional 48 hours, the transfected cells (about 4 x 10^4 cells/well) are washed with BM-NFDM, which is binding medium (RPMI 1640 containing 25 mg/ml bovine serum albumin, 2 mg/ml sodium azide, 20 mM Hepes pH 7.2) to which 50 mg/ml nonfat dry milk has been added. The cells then are incubated for 1 hour at 37°C with various concentrations of a soluble TRAIL-R/Fc fusion protein. Cells then are washed and incubated with a constant saturating concentration of a ^{125}I-mouse anti-human IgG in binding medium, with gentle agitation for 1 hour at 37°C. After extensive washing, cells are released *via* trypsinization.

The mouse anti-human IgG employed above is directed against the Fc region of human IgG and can be obtained from Jackson Immunoresearch Laboratories, Inc., West Grove, PA. The antibody is radioiodinated using the standard chloramine-T method. The antibody will bind to the Fc portion of any TRAIL-R/Fc protein that has bound to the cells. In all assays, non-specific binding of ^{125}I-antibody is assayed in the absence of

unlabeled mouse anti-human IgG antibody.

Cell-bound ^{125}I-antibody is quantified on a Packard Autogamma counter. Affinity calculations (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) are generated on RS/1 (BBN Software, Boston, MA) run on a Microvax computer.

Another type of suitable binding assay is a competitive binding assay. To illustrate, biological activity of a TRAIL-R variant may be determined by assaying for the variant's ability to compete with a native TRAIL-R for binding to TRAIL.

Competitive binding assays can be performed by conventional methodology. Reagents that may be employed in competitive binding assays include radiolabeled TRAIL-R and intact cells expressing TRAIL (endogenous or recombinant) on the cell surface. For example, a radiolabeled soluble TRAIL-R fragment can be used to compete with a soluble TRAIL-R variant for binding to cell surface TRAIL. Instead of intact cells, one could substitute a soluble TRAIL/Fc fusion protein bound to a solid phase through the interaction of Protein A or Protein G (on the solid phase) with the Fc moiety. Chromatography columns that contain Protein A and Protein G include those available from Pharmacia Biotech, Inc., Piscataway, NJ. Another type of competitive binding assay utilizes radiolabeled soluble TRAIL, such as a soluble TRAIL/Fc fusion protein, and intact cells expressing TRAIL-R. Qualitative results can be obtained by competitive autoradiographic plate binding assays, while Scatchard plots (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) may be utilized to generate quantitative results.

Another type of assay for biological activity involves testing a TRAIL-R polypeptide for the ability to block TRAIL-mediated apoptosis of target cells, such as the human leukemic T-cell line known as Jurkat cells, for example. TRAIL-mediated apoptosis of the cell line designated Jurkat clone E6-1 (ATCC TIB 152) is demonstrated in assay procedures described in PCT application WO 97/01633, hereby incorporated by reference.

Uses of TRAIL-R

Uses of TRAIL-R include, but are not limited to, the following. Certain of these uses of TRAIL-R flow from its ability to bind TRAIL.

TRAIL-R finds use as a protein purification reagent. TRAIL-R polypeptides may be attached to a solid support material and used to purify TRAIL proteins by affinity chromatography. In particular embodiments, a TRAIL-R polypeptide (in any form described herein that is capable of binding TRAIL) is attached to a solid support by conventional procedures. As one example, chromatography columns containing functional groups that will react with functional groups on amino acid side chains of proteins are available (Pharmacia Biotech, Inc., Piscataway, NJ). In an alternative, a

TRAIL-R/Fc protein is attached to Protein A- or Protein G-containing chromatography columns through interaction with the Fc moiety.

TRAIL-R proteins also find use in measuring the biological activity of TRAIL proteins in terms of their binding affinity for TRAIL-R. TRAIL-R proteins thus may be employed by those conducting "quality assurance" studies, e.g., to monitor shelf life and stability of TRAIL protein under different conditions. To illustrate, TRAIL-R may be employed in a binding affinity study to measure the biological activity of a TRAIL protein that has been stored at different temperatures, or produced in different cell types. TRAIL-R also may be used to determine whether biological activity is retained after modification of a TRAIL protein (e.g., chemical modification, truncation, mutation, etc.). The binding affinity of the modified TRAIL protein for TRAIL-R is compared to that of an unmodified TRAIL protein to detect any adverse impact of the modifications on biological activity of TRAIL. The biological activity of a TRAIL protein thus can be ascertained before it is used in a research study, for example.

TRAIL-R also finds use in purifying or identifying cells that express TRAIL on the cell surface. TRAIL-R polypeptides are bound to a solid phase such as a column chromatography matrix or a similar suitable substrate. For example, magnetic microspheres can be coated with TRAIL-R and held in an incubation vessel through a magnetic field. Suspensions of cell mixtures containing TRAIL-expressing cells are contacted with the solid phase having TRAIL-R thereon. Cells expressing TRAIL on the cell surface bind to the fixed TRAIL-R, and unbound cells then are washed away.

Alternatively, TRAIL-R can be conjugated to a detectable moiety, then incubated with cells to be tested for TRAIL expression. After incubation, unbound labeled TRAIL-R is removed and the presence or absence of the detectable moiety on the cells is determined.

In a further alternative, mixtures of cells suspected of containing TRAIL$^+$ cells are incubated with biotinylated TRAIL-R. Incubation periods are typically at least one hour in duration to ensure sufficient binding. The resulting mixture then is passed through a column packed with avidin-coated beads, whereby the high affinity of biotin for avidin provides binding of the desired cells to the beads. Procedures for using avidin-coated beads are known (see Berenson, et al. *J. Cell. Biochem.*, 10D:239, 1986). Washing to remove unbound material, and the release of the bound cells, are performed using conventional methods.

TRAIL-R polypeptides also find use as carriers for delivering agents attached thereto to cells bearing TRAIL. Cells expressing TRAIL include those identified in Wiley et al. (*Immunity*, 3:673-682, 1995). TRAIL-R proteins thus can be used to deliver diagnostic or therapeutic agents to such cells (or to other cell types found to express TRAIL on the cell surface) in *in vitro* or *in vivo* procedures.

Detectable (diagnostic) and therapeutic agents that may be attached to a TRAIL-R polypeptide include, but are not limited to, toxins, other cytotoxic agents, drugs, radionuclides, chromophores, enzymes that catalyze a colorimetric or fluorometric reaction, and the like, with the particular agent being chosen according to the intended application. Among the toxins are ricin, abrin, diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, ribosomal inactivating proteins, mycotoxins such as trichothecenes, and derivatives and fragments (e.g., single chains) thereof. Radionuclides suitable for diagnostic use include, but are not limited to, ^{123}I, ^{131}I, ^{99m}Tc, ^{111}In, and ^{76}Br. Examples of radionuclides suitable for therapeutic use are ^{131}I, ^{211}At, ^{77}Br, ^{186}Re, ^{188}Re, ^{212}Pb, ^{212}Bi, ^{109}Pd, ^{64}Cu, and ^{67}Cu.

Such agents may be attached to the TRAIL-R by any suitable conventional procedure. TRAIL-R, being a protein, comprises functional groups on amino acid side chains that can be reacted with functional groups on a desired agent to form covalent bonds, for example. Alternatively, the protein or agent may be derivatized to generate or attach a desired reactive functional group. The derivatization may involve attachment of one of the bifunctional coupling reagents available for attaching various molecules to proteins (Pierce Chemical Company, Rockford, Illinois). A number of techniques for radiolabeling proteins are known. Radionuclide metals may be attached to TRAIL-R by using a suitable bifunctional chelating agent, for example.

Conjugates comprising TRAIL-R and a suitable diagnostic or therapeutic agent (preferably covalently linked) are thus prepared. The conjugates are administered or otherwise employed in an amount appropriate for the particular application.

TRAIL-R DNA and polypeptides of the present invention may be used in developing treatments for any disorder mediated (directly or indirectly) by defective, or insufficient amounts of, TRAIL-R. TRAIL-R polypeptides may be administered to a mammal afflicted with such a disorder. Alternatively, a gene therapy approach may be taken. Disclosure herein of native TRAIL-R nucleotide sequences permits the detection of defective TRAIL-R genes, and the replacement thereof with normal TRAIL-R-encoding genes. Defective genes may be detected in *in vitro* diagnostic assays, and by comparison of a native TRAIL-R nucleotide sequence disclosed herein with that of a TRAIL-R gene derived from a person suspected of harboring a defect in this gene.

Another use of the protein of the present invention is as a research tool for studying the biological effects that result from inhibiting TRAIL/TRAIL-R interactions on different cell types. TRAIL-R polypeptides also may be employed in *in vitro* assays for detecting TRAIL or TRAIL-R or the interactions thereof.

A purified TRAIL-R polypeptide may be used to bind TRAIL, thus inhibiting the binding of TRAIL to endogenous cell surface TRAIL receptors. Such TRAIL receptors include the TRAIL-R disclosed herein, as well as TRAIL-binding proteins that are

distinct from the TRAIL-R of the present invention. Certain ligands of the TNF family (of which TRAIL is a member) have been reported to bind to more than one distinct cell surface receptor protein. TRAIL likewise may bind to multiple cell surface proteins. A receptor protein designated DR4 that reportedly binds TRAIL, but is distinct from the TRAIL-R of the present invention, is described in Pan et al. (*Science* 276:111-113, 1997; hereby incorporated by reference).

TRAIL-R may be used to inhibit a biological activity of TRAIL, in *in vitro* or *in vivo* procedures. By inhibiting binding of TRAIL to cell surface receptors, TRAIL-R consequently inhibits biological effects that result from the binding of TRAIL to endogenous receptors. Various forms of TRAIL-R may be employed, including, for example, the above-described TRAIL-R fragments, oligomers, derivatives, and variants that are capable of binding TRAIL. In a preferred embodiment, a soluble TRAIL-R is employed to inhibit a biological activity of TRAIL, e.g., to inhibit TRAIL-mediated apoptosis of cells susceptible to such apoptosis.

TRAIL-R may be administered to a mammal to treat a TRAIL-mediated disorder. Such TRAIL-mediated disorders include conditions caused (directly or indirectly) or exacerbated by TRAIL.

TRAIL-R may be useful for treating thrombotic microangiopathies. One such disorder is thrombotic thrombocytopenic purpura (TTP) (Kwaan, H.C., *Semin. Hematol.*, 24:71, 1987; Thompson et al., *Blood*, 80:1890, 1992). Increasing TTP-associated mortality rates have been reported by the U.S. Centers for Disease Control (Torok et al., *Am. J. Hematol.* 50:84, 1995).

Plasma from patients afflicted with TTP (including HIV^+ and HIV^- patients) induces apoptosis of human endothelial cells of dermal microvascular origin, but not large vessel origin (Laurence et al., *Blood*, 87:3245, April 15, 1996). Plasma of TTP patients thus is thought to contain one or more factors that directly or indirectly induce apoptosis. As described in PCT application WO 97/01633 (hereby incorporated by reference), TRAIL is present in the serum of TTP patients, and may play a role in inducing apoptosis of microvascular endothelial cells.

Another thrombotic microangiopathy is hemolytic-uremic syndrome (HUS) (Moake, J.L., *Lancet*, 343:393, 1994; Melnyk et al., (*Arch. Intern. Med.*, 155:2077, 1995; Thompson et al., *supra*). One embodiment of the invention is directed to the use of TRAIL-R to treat the condition that is often referred to as "adult HUS" (even though it can strike children as well). A disorder known as childhood/diarrhea-associated HUS differs in etiology from adult HUS.

Other conditions characterized by clotting of small blood vessels may be treated using TRAIL-R. Such conditions include but are not limited to the following. Cardiac problems seen in about 5-10% of pediatric AIDS patients are believed to involve clotting

in multiple sclerosis patients. As a further example, treatment of systemic lupus erythematosus (SLE) is contemplated.

In one embodiment, a patient's blood or plasma is contacted with TRAIL-R *ex vivo*. The TRAIL-R may be bound to a suitable chromatography matrix by conventional procedures. The patient's blood or plasma flows through a chromatography column containing TRAIL-R bound to the matrix, before being returned to the patient. The immobilized receptor binds TRAIL, thus removing TRAIL protein from the patient's blood.

Alternatively, TRAIL-R may be administered *in vivo* to a patient afflicted with a thrombotic microangiopathy. In one embodiment, a soluble form of TRAIL-R is administered to the patient.

The present invention thus provides a method for treating a thrombotic microangiopathy, involving use of an effective amount of TRAIL-R. A TRAIL-R polypeptide may be employed in *in vivo* or *ex vivo* procedures, to inhibit TRAIL-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

TRAIL-R may be employed in conjunction with other agents useful in treating a particular disorder. In an *in vitro* study reported by Laurence et al. (*Blood* 87:3245, 1996), some reduction of TTP plasma-mediated apoptosis of microvascular endothelial cells was achieved by using an anti-Fas blocking antibody, aurintricarboxylic acid, or normal plasma depleted of cryoprecipitate.

Thus, a patient may be treated with an agent that inhibits Fas-ligand-mediated apoptosis of endothelial cells, in combination with an agent that inhibits TRAIL-mediated apoptosis of endothelial cells. In one embodiment, TRAIL-R and an anti-FAS blocking antibody are both administered to a patient afflicted with a disorder characterized by thrombotic microangiopathy, such as TTP or HUS. Examples of blocking monoclonal antibodies directed against Fas antigen (CD95) are described in PCT application publication number WO 95/10540, hereby incorporated by reference.

Another embodiment of the present invention is directed to the use of TRAIL-R to reduce TRAIL-mediated death of T cells in HIV-infected patients. The role of T cell apoptosis in the development of AIDS has been the subject of a number of studies (see, for example, Meyaard et al., *Science* 257:217-219, 1992; Groux et al., *J Exp. Med.*, 175:331, 1992; and Oyaizu et al., in *Cell Activation and Apoptosis in HIV Infection*, Andrieu and Lu, Eds., Plenum Press, New York, 1995, pp. 101-114). Certain investigators have studied the role of Fas-mediated apoptosis; the involvement of interleukin-1ß-converting enzyme (ICE) also has been explored (Estaquier et al., *Blood* 87:4959-4966, 1996; Mitra et al., *Immunology* 87:581-585, 1996; Katsikis et al., *J. Exp.*

Med. 181:2029-2036, 1995). It is possible that T cell apoptosis occurs through multiple mechanisms.

At least some of the T cell death seen in HIV$^+$ patients is believed to be mediated by TRAIL. While not wishing to be bound by theory, such TRAIL-mediated T cell death is believed to occur through the mechanism known as activation-induced cell death (AICD).

Activated human T cells are induced to undergo programmed cell death (apoptosis) upon triggering through the CD3/T cell receptor complex, a process termed activated-induced cell death (AICD). AICD of CD4$^+$ T cells isolated from HIV-infected asymptomatic individuals has been reported (Groux et al., *supra*). Thus, AICD may play a role in the depletion of CD4$^+$ T cells and the progression to AIDS in HIV-infected individuals.

The present invention provides a method of inhibiting TRAIL-mediated T cell death in HIV$^+$ patients, comprising administering TRAIL-R (preferably, a soluble TRAIL-R polypeptide) to the patients. In one embodiment, the patient is asymptomatic when treatment with TRAIL-R commences. If desired, prior to treatment, peripheral blood T cells may be extracted from an HIV$^+$ patient, and tested for susceptibility to TRAIL-mediated cell death by conventional procedures.

In one embodiment, a patient's blood or plasma is contacted with TRAIL-R *ex vivo*. The TRAIL-R may be bound to a suitable chromatography matrix by conventional procedures. The patient's blood or plasma flows through a chromatography column containing TRAIL-R bound to the matrix, before being returned to the patient. The immobilized TRAIL-R binds TRAIL, thus removing TRAIL protein from the patient's blood.

In treating HIV$^+$ patients, TRAIL-R may be employed in combination with other inhibitors of T cell apoptosis. Fas-mediated apoptosis also has been implicated in loss of T cells in HIV$^+$ individuals (Katsikis et al., *J. Exp. Med.* 181:2029-2036, 1995). Thus, a patient susceptible to both Fas ligand (Fas-L)-mediated and TRAIL-mediated T cell death may be treated with both an agent that blocks TRAIL/TRAIL receptor interactions and an agent that blocks Fas-L/Fas interactions. Suitable agents for blocking binding of Fas-L to Fas include, but are not limited to, soluble Fas polypeptides; oligomeric forms of soluble Fas polypeptides (e.g., dimers of sFas/Fc); anti-Fas antibodies that bind Fas without transducing the biological signal that results in apoptosis; anti-Fas-L antibodies that block binding of Fas-L to Fas; and muteins of Fas-L that bind Fas but don't transduce the biological signal that results in apoptosis. Preferably, the antibodies employed in the method are monoclonal antibodies. Examples of suitable agents for blocking Fas-L/Fas interactions, including blocking anti-Fas monoclonal antibodies, are described in WO 95/10540, hereby incorporated by reference.

Compositions comprising an effective amount of a TRAIL-R polypeptide of the present invention, in combination with other components such as a physiologically acceptable diluent, carrier, or excipient, are provided herein. TRAIL-R can be formulated according to known methods used to prepare pharmaceutically useful compositions. TRAIL-R can be combined in admixture, either as the sole active material or with other known active materials suitable for a given indication, with pharmaceutically acceptable diluents (e.g., saline, Tris-HCl, acetate, and phosphate buffered solutions), preservatives (e.g., thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable formulations for pharmaceutical compositions include those described in *Remington's Pharmaceutical Sciences*, 16th ed. 1980, Mack Publishing Company, Easton, PA.

In addition, such compositions can contain TRAIL-R complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of TRAIL-R, and are thus chosen according to the intended application. TRAIL-R expressed on the surface of a cell may find use, as well.

Compositions of the present invention may contain a TRAIL-R polypeptide in any form described herein, such as native proteins, variants, derivatives, oligomers, and biologically active fragments. In particular embodiments, the composition comprises a soluble TRAIL-R polypeptide or an oligomer comprising soluble TRAIL-R polypeptides.

TRAIL-R can be administered in any suitable manner, e.g., topically, parenterally, or by inhalation. The term "parenteral" includes injection, e.g., by subcutaneous, intravenous, or intramuscular routes, also including localized administration. Sustained release from implants is also contemplated. One skilled in the pertinent art will recognize that suitable dosages will vary, depending upon such factors as the nature of the disorder to be treated, the patient's body weight, age, and general condition, and the route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration are performed according to art-accepted practices.

Compositions comprising TRAIL-R nucleic acids in physiologically acceptable formulations are also contemplated. TRAIL-R DNA may be formulated for injection, for example.

Antibodies

Antibodies that bind TRAIL-R polypeptides are provided herein. The TRAIL-R protein of Figure 1 or 2 (SEQ ID NO:2 or 4) may be employed as an immunogen in producing antibodies immunoreactive therewith. Alternatively, another form of TRAIL-R, such as a fragment or fusion protein, is employed as the immunogen.

The present invention thus provides antibodies obtained by immunizing an animal with the TRAIL-R of Figure 1 or 2, or an immunogenic fragment thereof. A method for producing antibodies comprises immunizing an animal with a TRAIL-R polypeptide, whereby antibodies directed against the TRAIL-R are generated in said animal. The desired antibodies may be purified, e.g., from the animal's serum, by conventional techniques.

Among the procedures for preparing polyclonal and monoclonal antibodies are those described in *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet et al. (eds.), Plenum Press, New York (1980); and *Antibodies: A Laboratory Manual* , Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1988). Production of monoclonal antibodies directed against TRAIL-R is further illustrated in example 4.

Antigen-binding fragments of such antibodies, which may be produced by conventional techniques, are also encompassed by the present invention. Examples of such fragments include, but are not limited to, Fab and $F(ab')_2$ fragments. Antibody fragments and derivatives produced by genetic engineering techniques are also provided.

The monoclonal antibodies of the present invention include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to humans. In one embodiment, a humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann et al. (*Nature 332*:323, 1988), Liu et al. (*PNAS 84*:3439, 1987), Larrick et al. (*Bio/Technology 7*:934, 1989), and Winter and Harris (*TIPS 14*:139, May, 1993).

In one embodiment, the antibodies are specific for the TRAIL-R of the present invention, and do not cross-react with other (non-TRAIL-R) proteins. Screening procedures by which such antibodies may be identified are well known, and may involve immunoaffinity chromatography, for example.

Hybridoma cell lines that produce monoclonal antibodies specific for TRAIL-R are also contemplated herein. Such hybridomas may be produced and identified by conventional techniques. One method for producing such a hybridoma cell line comprises immunizing an animal with a TRAIL-R; harvesting spleen cells from the immunized animal; fusing said spleen cells to a myeloma cell line, thereby generating hybridoma cells; and identifying a hybridoma cell line that produces a monoclonal antibody that binds TRAIL-R. The monoclonal antibodies may be recovered by conventional techniques.

Among the uses of the antibodies is use in assays to detect the presence of TRAIL-R polypeptides, either *in vitro* or *in vivo*. The antibodies also may be employed in purifying TRAIL-R proteins by immunoaffinity chromatography.

In one embodiment, the antibodies additionally can block binding of TRAIL to TRAIL-R. Such antibodies may be employed to inhibit binding of TRAIL to cell surface TRAIL-R, for example. Blocking antibodies may be identified using conventional assay procedures.

Such an antibody may be employed in an *in vitro* procedure, or administered *in vivo* to inhibit a TRAIL-R-mediated biological activity. Disorders caused or exacerbated (directly or indirectly) by the interaction of TRAIL with cell surface TRAIL-R thus may be treated. A therapeutic method involves *in vivo* administration of a blocking antibody to a mammal in an amount effective in inhibiting a TRAIL-mediated biological activity. Disorders caused or exacerbated by TRAIL, directly or indirectly, are thus treated. Monoclonal antibodies are generally preferred for use in such therapeutic methods. In one embodiment, an antigen-binding antibody fragment is employed.

Compositions comprising an antibody that is directed against TRAIL-R, and a physiologically acceptable diluent, excipient, or carrier, are provided herein. Suitable components of such compositions are as described above for compositions containing TRAIL-R proteins.

Also provided herein are conjugates comprising a detectable (e.g., diagnostic) or therapeutic agent, attached to an antibody directed against TRAIL-R. Examples of such agents are presented above. The conjugates find use in *in vitro* or *in vivo* procedures.

Nucleic Acids

The present invention provides TRAIL-R nucleic acids. Such nucleic acids include, but are not limited to, the human TRAIL-R DNA of Figures 1 and 2 (SEQ ID NOS:1 and 3). Nucleic acid molecules of the present invention include TRAIL-R DNA in both single-stranded and double-stranded form, as well as the RNA complement thereof. TRAIL-R DNA includes, for example, cDNA, genomic DNA, chemically synthesized DNA, DNA amplified by PCR, and combinations thereof. Genomic DNA

may be isolated by conventional techniques, e.g., using the cDNA of Figures 1 or 2, or a suitable fragment thereof, as a probe.

In particular embodiments, the nucleic acids are useful in the production of TRAIL-R polypeptides, e.g., in the recombinant expression systems discussed above. DNAs encoding TRAIL-R in any of the forms contemplated herein (e.g., full length TRAIL-R or fragments thereof) are provided. Particular embodiments of TRAIL-R-encoding DNAs include DNA encoding the full length human TRAIL-R of Figures 1 or 2 (including the N-terminal signal peptide), and DNA encoding full length mature human TRAIL-R. Other embodiments include DNA encoding soluble TRAIL-R (e.g., encoding the extracellular domain of the protein, either with or without the signal peptide).

TRAIL-R nucleic acid fragments provided herein have uses that include, but are not limited to, use as probes or primers. Oligonucleotides derived from the nucleic acid sequences disclosed herein may be employed as 3' and 5' primers in polymerase chain reactions (PCR), for example, whereby TRAIL-R DNA fragments are isolated and amplified.

Particular fragments of TRAIL-R nucleotide sequences comprise at least about 30, or at least 60, contiguous nucleotides of a TRAIL-R DNA sequence. Nucleic acids provided herein include DNA and RNA complements of said fragments, along with both single-stranded and double-stranded forms of the TRAIL-R DNA.

Other useful fragments of the TRAIL-R nucleic acids include antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target TRAIL-R mRNA (sense) or TRAIL-R DNA (antisense) sequences. Antisense or sense oligonucleotides, according to the present invention, may comprise a fragment of the coding region of the TRAIL-R DNA of Figure 1 or 2. Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to about 30 nucleotides. The ability to derive an antisense or a sense oligonucleotide, based upon a cDNA sequence encoding a given protein is described in, for example, Stein and Cohen (*Cancer Res.* 48:2659, 1988) and van der Krol et al. (*BioTechniques* 6:958, 1988).

Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block transcription or translation of the target sequence by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. The antisense oligonucleotides thus may be used to block expression of TRAIL-R proteins. Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of

resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences.

Other examples of sense or antisense olig nucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10448, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oligonucleotide for the target nucleotide sequence.

Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, $CaPO_4$-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus. In a preferred procedure, an antisense or sense oligonucleotide is inserted into a suitable retroviral vector. A cell containing the target nucleic acid sequence is contacted with the recombinant retroviral vector, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, those derived from the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or the double copy vectors designated DCT5A, DCT5B and DCT5C (see WO 90/13641).

Sense or antisense oligonucleotides also may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

The following examples are provided to further illustrate particular embodiments of the invention, and are not to be construed as limiting the scope of the present invention.

EXAMPLE 1: TRAIL-R cDNA Clones

TRAIL-R clones were isolated from two cDNA libraries. The first cDNA library was derived from human foreskin fibroblasts; the second was derived from human peripheral blood lymphocytes (PBLs).

A TRAIL-R cDNA clone isolated from the PBL library was found to comprise the nucleotide sequence shown in Figure 2 (SEQ ID NO:3). Both forms of TRAIL-R (the sequence presented in Figure 1 or Figure 2) were represented in clones isolated from the human foreskin fibroblast library.

The nucleotide sequences of Figure 1 (SEQ ID NO:1) and Figure 2 (SEQ ID NO:3) differ at two positions. In Figure 1, the nucleotide at position 145 is a C, whereas nucleotide 145 is a T in Figure 2; the nucleotide at position 971 is a C in Figure 1, but is a T in Figure 2. The amino acid sequences likewise differ at two positions. Residue 35 is Pro in Figure 1 (SEQ ID NO:2) and Ser in Figure 2 (SEQ ID NO:4); residue 310 is Ser in Figure 1 and Leu in Figure 2. One possible explanation is that the TRAIL receptors of Figures 1 and 2 are allelic variants.

The TRAIL-R proteins of Figure 1 (SEQ ID NO:2) and Figure 2 (SEQ ID NO:4) include an N-terminal hydrophobic region that functions as a signal peptide. One signal peptide cleavage site predicted by computer analysis follows amino acid 55 of Figures 1 and 2. Cleavage of the signal peptide (amino acids 1 to 55) thus would yield a mature protein comprising amino acids 56 through 386. The protein further comprises an extracellular domain (amino acids 56 to 211), a transmembrane region (amino acids 212 through 232), and a C-terminal cytoplasmic domain (amino acids 233 through 386).

The two amino acids that differ in the proteins of Figures 1 and 2 are found in the signal peptide (at position 35) and in the cytoplasmic domain (at position 310). The extracellular domains of the proteins of Figures 1 and 2 thus are identical.

EXAMPLE 2: Binding Assay

TRAIL-R was tested for the ability to bind TRAIL, in a slide binding assay. DNA encoding the full length TRAIL-R of Figure 1 was inserted into a mammalian expression vector designated pDC409. pDC409 was derived from the pDC406 vector described in McMahan et al. (*EMBO J.* 10:2821-2832, 1991; hereby incorporated by reference). Features added to pDC409 (compared to pDC406) include additional unique restriction sites in the multiple cloning site (mcs); three stop codons (one in each reading frame) positioned downstream of the mcs; and a T7 polymerase promoter, downstream of the mcs, that faciliates sequencing of DNA inserted into the mcs.

CV-1/EBNA cells were transfected with the recombinant expression vector, and cultured on glass slides to allow expression of TRAIL-R. A slide binding assay was conducted generally as described in Gearing et al. (*EMBO J.* 8:3667, 1989); McMahan et al. (*EMBO J.* 10:2821, 1991) and Goodwin et al. (*Eur. J. Immunol.* 23:2631, 1993), hereby incorporated by reference. Briefly, the transfected cells were incubated with an

LZ-TRAIL fusion protein (described below) in binding media. Slides were then washed, and an ^{125}I-labeled antibody specific for the leucine zipper (LZ) moiety of the fusion protein was added. After incubation with the antibody, the slides were washed, fixed, and dipped in photographic emulsion. The assay demonstrated that the TRAIL-R protein binds TRAIL.

The LZ-TRAIL employed in the assay is a fusion protein comprising a leucine zipper peptide fused to the N-terminus of a soluble TRAIL polypeptide (LZ-TRAIL) was employed in the assay. An expression construct was prepared, essentially as described for preparation of the Flag®-TRAIL expression construct in Wiley et al. (*Immunity*, 3:673-682, 1995; hereby incorporated by reference), except that DNA encoding the Flag® peptide was replaced with a sequence encoding a modified leucine zipper that allows for trimerization. The construct, in expression vector pDC409, encoded a leader sequence derived from human cytomegalovirus, followed by the leucine zipper moiety fused to the N-terminus of a soluble TRAIL polypeptide. The TRAIL polypeptide comprised amino acids 95-281 of human TRAIL (a fragment of the extracellular domain), as described in Wiley et al. (*supra*). The LZ-TRAIL was expressed in CHO cells, and purified from the culture supernatant.

EXAMPLE 3: Preparation of a Soluble TRAIL-R

An expression vector encoding a soluble TRAIL-R/Fc fusion protein was constructed, as follows. The fusion protein comprised a soluble TRAIL-R polypeptide fused to the N-terminus of an IgG1 Fc region polypeptide mutein.

A DNA fragment encoding amino acids 1 to 208 of Figure 1 (SEQ ID NO:2) was isolated by polymerase chain reaction (PCR). Oligonucleotides that defined the desired termini of the DNA fragment were employed as the 3' and 5' primers in the PCR, and the cDNA clone represented in Figure 1 (SEQ ID NO:1) was employed as the template.

The Fc moiety of the fusion protein was a mutein of a human IgG1 Fc region polypeptide. DNA and amino acid sequence information for this Fc mutein are described in U.S. Patent 5,457,035 and in Baum et al., (*EMBO J.* 13:3992-4001, 1994), hereby incorporated by reference.

The procedures for preparing an expression vector containing a TRAIL-R/Fc gene fusion were generally as described in Smith et al. (*Cell* 73:1349-1360, 1993) and Fanslow et al. (*J. Immunol.* 149:655-660, 1992), which are hereby incorporated by reference. The

expression vector pDC409, described in example 2, was employed. CV1/EBNA cells were transfected with the resulting recombinant expression vector, and cultured to allow expression and secretion of the TRAIL-R/Fc from the cells.

EXAMPLE 4: Monoclonal Antibodies That Bind TRAIL-R

This example illustrates a method for preparing monoclonal antibodies that bind TRAIL-R. Suitable immunogens that may be employed in generating such antibodies include, but are not limited to, purified TRAIL-R protein or an immunogenic fragment thereof such as the extracellular domain, or fusion proteins containing TRAIL-R (e.g., a soluble TRAIL-R/Fc fusion protein).

Purified TRAIL-R can be used to generate monoclonal antibodies immunoreactive therewith, using conventional techniques such as those described in U.S. Patent 4,411,993. Briefly, mice are immunized with TRAIL-R immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 μg subcutaneously or intraperitoneally. Ten to twelve days later, the immunized animals are boosted with additional TRAIL-R emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision to test for TRAIL-R antibodies by dot blot assay, ELISA (Enzyme-Linked Immunosorbent Assay) or inhibition of TRAIL binding.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of TRAIL-R in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line, e.g., NS1 or preferably P3x63Ag8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified TRAIL-R by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described in Beckmann et al., (*J. Immunol.* 144:4212, 1990) Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-TRAIL-R monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various

techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to Protein A or Protein G can also be used, as can affinity chromatography based upon binding to TRAIL-R.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: IMMUNEX CORPORATION.

(ii) TITLE OF INVENTION: TRAIL RECEPTOR

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Kathryn A. Anderson, Immunex Corporation
(B) STREET: 51 University Street
(C) CITY: Seattle
(D) STATE: WA
(E) COUNTRY: US
(F) ZIP: 98101

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: MS-DOS/Windows 95
(D) SOFTWARE: Word for Windows 95, 7.0a

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: --to be assigned--
(B) FILING DATE: 10-JUL-1998
(C) CLASSIFICATION

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/892,119
(B) FILING DATE: 15-JUL-1997
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Anderson, Kathryn A.
(B) REGISTRATION NUMBER: 32,172
(C) REFERENCE/DOCKET NUMBER: 2630-WO

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 206-587-0430
(B) TELEFAX: 206-233-0644

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1552 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: TRAILR4A

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 43..1203

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TGATTGATTT TTGGCGCTTT CGATCCACCC TCCTCCCTTC TC ATG GGA CTT TGG          54
                                                 Met Gly Leu Trp
                                                   1

GGA CAA AGC GTC CCG ACC GCC TCG AGC GCT CGA GCA GGG CGC TAT CCA         102
Gly Gln Ser Val Pro Thr Ala Ser Ser Ala Arg Ala Gly Arg Tyr Pro
  5                  10                  15                  20

GGA GCC AGG ACA GCG TCG GGA ACC AGA CCA TGG CTC CTG GAC CCC AAG         150
Gly Ala Arg Thr Ala Ser Gly Thr Arg Pro Trp Leu Leu Asp Pro Lys
                  25                  30                  35

ATC CTT AAG TTC GTC GTC TTC ATC GTC GCG GTT CTG CTG CCG GTC CGG         198
Ile Leu Lys Phe Val Val Phe Ile Val Ala Val Leu Leu Pro Val Arg
              40                  45                  50

GTT GAC TCT GCC ACC ATC CCC CGG CAG GAC GAA GTT CCC CAG CAG ACA         246
Val Asp Ser Ala Thr Ile Pro Arg Gln Asp Glu Val Pro Gln Gln Thr
          55                  60                  65

GTG GCC CCA CAG CAA CAG AGG CGC AGC CTC AAG GAG GAG GAG TGT CCA         294
Val Ala Pro Gln Gln Gln Arg Arg Ser Leu Lys Glu Glu Glu Cys Pro
      70                  75                  80

GCA GGA TCT CAT AGA TCA GAA TAT ACT GGA GCC TGT AAC CCG TGC ACA         342
Ala Gly Ser His Arg Ser Glu Tyr Thr Gly Ala Cys Asn Pro Cys Thr
  85                  90                  95                  100

GAG GGT GTG GAT TAC ACC ATT GCT TCC AAC AAT TTG CCT TCT TGC CTG         390
Glu Gly Val Asp Tyr Thr Ile Ala Ser Asn Asn Leu Pro Ser Cys Leu
                 105                 110                 115

CTA TGT ACA GTT TGT AAA TCA GGT CAA ACA AAT AAA AGT TCC TGT ACC         438
Leu Cys Thr Val Cys Lys Ser Gly Gln Thr Asn Lys Ser Ser Cys Thr
             120                 125                 130

ACG ACC AGA GAC ACC GTG TGT CAG TGT GAA AAA GGA AGC TTC CAG GAT         486
Thr Thr Arg Asp Thr Val Cys Gln Cys Glu Lys Gly Ser Phe Gln Asp
         135                 140                 145

AAA AAC TCC CCT GAG ATG TGC CGG ACG TGT AGA ACA GGG TGT CCC AGA         534
Lys Asn Ser Pro Glu Met Cys Arg Thr Cys Arg Thr Gly Cys Pro Arg
     150                 155                 160

GGG ATG GTC AAG GTC AGT AAT TGT ACG CCC CGG AGT GAC ATC AAG TGC         582
Gly Met Val Lys Val Ser Asn Cys Thr Pro Arg Ser Asp Ile Lys Cys
165                 170                 175                 180

AAA AAT GAA TCA GCT GCC AGT TCC ACT GGG AAA ACC CCA GCA GCG GAG         630
Lys Asn Glu Ser Ala Ala Ser Ser Thr Gly Lys Thr Pro Ala Ala Glu
                 185                 190                 195

GAG ACA GTG ACC ACC ATC CTG GGG ATG CTT GCC TCT CCC TAT CAC TAC         678
Glu Thr Val Thr Thr Ile Leu Gly Met Leu Ala Ser Pro Tyr His Tyr
             200                 205                 210

CTT ATC ATC ATA GTG GTT TTA GTC ATC ATT TTA GCT GTG GTT GTG GTT         726
Leu Ile Ile Ile Val Val Leu Val Ile Ile Leu Ala Val Val Val Val
         215                 220                 225

GGC TTT TCA TGT CGG AAG AAA TTC ATT TCT TAC CTC AAA GGC ATC TGC         774
Gly Phe Ser Cys Arg Lys Lys Phe Ile Ser Tyr Leu Lys Gly Ile Cys
     230                 235                 240
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TCA GGT GGT GGA GGA GGT CCC GAA CGT GTG CAC AGA GTC CTT TTC CGG      822
Ser Gly Gly Gly Gly Gly Pro Glu Arg Val His Arg Val Leu Phe Arg
245                 250                 255                 260

CGG CGT TCA TGT CCT TCA CGA GTT CCT GGG GCG GAG GAC AAT GCC CGC      870
Arg Arg Ser Cys Pro Ser Arg Val Pro Gly Ala Glu Asp Asn Ala Arg
                265                 270                 275

AAC GAG ACC CTG AGT AAC AGA TAC TTG CAG CCC ACC CAG GTC TCT GAG      918
Asn Glu Thr Leu Ser Asn Arg Tyr Leu Gln Pro Thr Gln Val Ser Glu
            280                 285                 290

CAG GAA ATC CAA GGT CAG GAG CTG GCA GAG CTA ACA GGT GTG ACT GTA      966
Gln Glu Ile Gln Gly Gln Glu Leu Ala Glu Leu Thr Gly Val Thr Val
        295                 300                 305

GAG TCG CCA GAG GAG CCA CAG CGT CTG CTG GAA CAG GCA GAA GCT GAA     1014
Glu Ser Pro Glu Glu Pro Gln Arg Leu Leu Glu Gln Ala Glu Ala Glu
    310                 315                 320

GGG TGT CAG AGG AGG AGG CTG CTG GTT CCA GTG AAT GAC GCT GAC TCC     1062
Gly Cys Gln Arg Arg Arg Leu Leu Val Pro Val Asn Asp Ala Asp Ser
325                 330                 335                 340

GCT GAC ATC AGC ACC TTG CTG GAT GCC TCG GCA ACA CTG GAA GAA GGA     1110
Ala Asp Ile Ser Thr Leu Leu Asp Ala Ser Ala Thr Leu Glu Glu Gly
                345                 350                 355

CAT GCA AAG GAA ACA ATT CAG GAC CAA CTG GTG GGC TCC GAA AAG CTC     1158
His Ala Lys Glu Thr Ile Gln Asp Gln Leu Val Gly Ser Glu Lys Leu
            360                 365                 370

TTT TAT GAA GAA GAT GAG GCA GGC TCT GCT ACG TCC TGC CTG TGA         1203
Phe Tyr Glu Glu Asp Glu Ala Gly Ser Ala Thr Ser Cys Leu  *
        375                 380                 385

AAGAATCTCT TCAGGAAACC AGAGCTTCCC TCATTTACCT TTTCTCCTAC AAAGGGAAGC  1263

AGCCTGGAAG AAACAGTCCA GTACTTGACC CATGCCCCAA CAAACTCTAC TATCCAATAT  1323

GGGGCAGCTT ACCAATGGTC CTAGAACTTT GTTAACGCAC TTGGAGTAAT TTTTATGAAA  1383

TACTGCGTGT GATAAGCAAA CGGGAGAAAT TTATATCAGA TTCTTGGCTG CATAGTTATA  1443

CGATTGTGTA TTAAGGGTCG TTTTAGGCCA CATGCGGTGG CTCATGCCTG TAATCCCAGC  1503

ACTTTGATAG GCTGAGGCAG GTGGATTGCT TGAGCTCGGG AGTTTGAGA              1552
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 386 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Gly Leu Trp Gly Gln Ser Val Pro Thr Ala Ser Ser Ala Arg Ala
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Gly Arg Tyr Pro Gly Ala Arg Thr Ala Ser Gly Thr Arg Pro Trp Leu
                20                  25                  30

Leu Asp Pro Lys Ile Leu Lys Phe Val Val Phe Ile Val Ala Val Leu
            35                  40                  45

Leu Pro Val Arg Val Asp Ser Ala Thr Ile Pro Arg Gln Asp Glu Val
        50                  55                  60

Pro Gln Gln Thr Val Ala Pro Gln Gln Gln Arg Arg Ser Leu Lys Glu
    65                  70                  75                  80

Glu Glu Cys Pro Ala Gly Ser His Arg Ser Glu Tyr Thr Gly Ala Cys
                85                  90                  95

Asn Pro Cys Thr Glu Gly Val Asp Tyr Thr Ile Ala Ser Asn Asn Leu
            100                 105                 110

Pro Ser Cys Leu Leu Cys Thr Val Cys Lys Ser Gly Gln Thr Asn Lys
        115                 120                 125

Ser Ser Cys Thr Thr Thr Arg Asp Thr Val Cys Gln Cys Glu Lys Gly
    130                 135                 140

Ser Phe Gln Asp Lys Asn Ser Pro Glu Met Cys Arg Thr Cys Arg Thr
145                 150                 155                 160

Gly Cys Pro Arg Gly Met Val Lys Val Ser Asn Cys Thr Pro Arg Ser
                165                 170                 175

Asp Ile Lys Cys Lys Asn Glu Ser Ala Ala Ser Ser Thr Gly Lys Thr
            180                 185                 190

Pro Ala Ala Glu Glu Thr Val Thr Thr Ile Leu Gly Met Leu Ala Ser
        195                 200                 205

Pro Tyr His Tyr Leu Ile Ile Ile Val Val Leu Val Ile Ile Leu Ala
    210                 215                 220

Val Val Val Val Gly Phe Ser Cys Arg Lys Lys Phe Ile Ser Tyr Leu
225                 230                 235                 240

Lys Gly Ile Cys Ser Gly Gly Gly Gly Gly Pro Glu Arg Val His Arg
                245                 250                 255

Val Leu Phe Arg Arg Arg Ser Cys Pro Ser Arg Val Pro Gly Ala Glu
            260                 265                 270

Asp Asn Ala Arg Asn Glu Thr Leu Ser Asn Arg Tyr Leu Gln Pro Thr
        275                 280                 285

Gln Val Ser Glu Gln Glu Ile Gln Gly Gln Glu Leu Ala Glu Leu Thr
    290                 295                 300

Gly Val Thr Val Glu Ser Pro Glu Glu Pro Gln Arg Leu Leu Glu Gln
305                 310                 315                 320

Ala Glu Ala Glu Gly Cys Gln Arg Arg Arg Leu Leu Val Pro Val Asn
                325                 330                 335

Asp Ala Asp Ser Ala Asp Ile Ser Thr Leu Leu Asp Ala Ser Ala Thr
            340                 345                 350

Leu Glu Glu Gly His Ala Lys Glu Thr Ile Gln Asp Gln Leu Val Gly
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        355                 360                 365

Ser Glu Lys Leu Phe Tyr Glu Glu Asp Glu Ala Gly Ser Ala Thr Ser
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Cys Leu  *
385
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1296 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: TRAILR4B

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 43..1203

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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                                                 Met Gly Leu Trp
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GGA CAA AGC GTC CCG ACC GCC TCG AGC GCT CGA GCA GGG CGC TAT CCA     102
Gly Gln Ser Val Pro Thr Ala Ser Ser Ala Arg Ala Gly Arg Tyr Pro
  5                  10                  15                  20

GGA GCC AGG ACA GCG TCG GGA ACC AGA CCA TGG CTC CTG GAC TCC AAG     150
Gly Ala Arg Thr Ala Ser Gly Thr Arg Pro Trp Leu Leu Asp Ser Lys
                 25                  30                  35

ATC CTT AAG TTC GTC GTC TTC ATC GTC GCG GTT CTG CTG CCG GTC CGG     198
Ile Leu Lys Phe Val Val Phe Ile Val Ala Val Leu Leu Pro Val Arg
             40                  45                  50

GTT GAC TCT GCC ACC ATC CCC CGG CAG GAC GAA GTT CCC CAG CAG ACA     246
Val Asp Ser Ala Thr Ile Pro Arg Gln Asp Glu Val Pro Gln Gln Thr
         55                  60                  65

GTG GCC CCA CAG CAA CAG AGG CGC AGC CTC AAG GAG GAG GAG TGT CCA     294
Val Ala Pro Gln Gln Gln Arg Arg Ser Leu Lys Glu Glu Glu Cys Pro
     70                  75                  80

GCA GGA TCT CAT AGA TCA GAA TAT ACT GGA GCC TGT AAC CCG TGC ACA     342
Ala Gly Ser His Arg Ser Glu Tyr Thr Gly Ala Cys Asn Pro Cys Thr
 85                  90                  95                 100

GAG GGT GTG GAT TAC ACC ATT GCT TCC AAC AAT TTG CCT TCT TGC CTG     390
Glu Gly Val Asp Tyr Thr Ile Ala Ser Asn Asn Leu Pro Ser Cys Leu
                105                 110                 115

CTA TGT ACA GTT TGT AAA TCA GGT CAA ACA AAT AAA AGT TCC TGT ACC     438
Leu Cys Thr Val Cys Lys Ser Gly Gln Thr Asn Lys Ser Ser Cys Thr
            120                 125                 130
```

```
ACG ACC AGA GAC ACC GTG TGT CAG TGT GAA AAA GGA AGC TTC CAG GAT      486
Thr Thr Arg Asp Thr Val Cys Gln Cys Glu Lys Gly Ser Phe Gln Asp
        135                 140                 145

AAA AAC TCC CCT GAG ATG TGC CGG ACG TGT AGA ACA GGG TGT CCC AGA      534
Lys Asn Ser Pro Glu Met Cys Arg Thr Cys Arg Thr Gly Cys Pro Arg
    150                 155                 160

GGG ATG GTC AAG GTC AGT AAT TGT ACG CCC CGG AGT GAC ATC AAG TGC      582
Gly Met Val Lys Val Ser Asn Cys Thr Pro Arg Ser Asp Ile Lys Cys
165                 170                 175                 180

AAA AAT GAA TCA GCT GCC AGT TCC ACT GGG AAA ACC CCA GCA GCG GAG      630
Lys Asn Glu Ser Ala Ala Ser Ser Thr Gly Lys Thr Pro Ala Ala Glu
                185                 190                 195

GAG ACA GTG ACC ACC ATC CTG GGG ATG CTT GCC TCT CCC TAT CAC TAC      678
Glu Thr Val Thr Thr Ile Leu Gly Met Leu Ala Ser Pro Tyr His Tyr
            200                 205                 210

CTT ATC ATC ATA GTG GTT TTA GTC ATC ATT TTA GCT GTG GTT GTG GTT      726
Leu Ile Ile Ile Val Val Leu Val Ile Ile Leu Ala Val Val Val Val
        215                 220                 225

GGC TTT TCA TGT CGG AAG AAA TTC ATT TCT TAC CTC AAA GGC ATC TGC      774
Gly Phe Ser Cys Arg Lys Lys Phe Ile Ser Tyr Leu Lys Gly Ile Cys
    230                 235                 240

TCA GGT GGT GGA GGA GGT CCC GAA CGT GTG CAC AGA GTC CTT TTC CGG      822
Ser Gly Gly Gly Gly Gly Pro Glu Arg Val His Arg Val Leu Phe Arg
245                 250                 255                 260

CGG CGT TCA TGT CCT TCA CGA GTT CCT GGG GCG GAG GAC AAT GCC CGC      870
Arg Arg Ser Cys Pro Ser Arg Val Pro Gly Ala Glu Asp Asn Ala Arg
                265                 270                 275

AAC GAG ACC CTG AGT AAC AGA TAC TTG CAG CCC ACC CAG GTC TCT GAG      918
Asn Glu Thr Leu Ser Asn Arg Tyr Leu Gln Pro Thr Gln Val Ser Glu
            280                 285                 290

CAG GAA ATC CAA GGT CAG GAG CTG GCA GAG CTA ACA GGT GTG ACT GTA      966
Gln Glu Ile Gln Gly Gln Glu Leu Ala Glu Leu Thr Gly Val Thr Val
        295                 300                 305

GAG TTG CCA GAG GAG CCA CAG CGT CTG CTG GAA CAG GCA GAA GCT GAA     1014
Glu Leu Pro Glu Glu Pro Gln Arg Leu Leu Glu Gln Ala Glu Ala Glu
    310                 315                 320

GGG TGT CAG AGG AGG AGG CTG CTG GTT CCA GTG AAT GAC GCT GAC TCC     1062
Gly Cys Gln Arg Arg Arg Leu Leu Val Pro Val Asn Asp Ala Asp Ser
325                 330                 335                 340

GCT GAC ATC AGC ACC TTG CTG GAT GCC TCG GCA ACA CTG GAA GAA GGA     1110
Ala Asp Ile Ser Thr Leu Leu Asp Ala Ser Ala Thr Leu Glu Glu Gly
                345                 350                 355

CAT GCA AAG GAA ACA ATT CAG GAC CAA CTG GTG GGC TCC GAA AAG CTC     1158
His Ala Lys Glu Thr Ile Gln Asp Gln Leu Val Gly Ser Glu Lys Leu
            360                 365                 370

TTT TAT GAA GAA GAT GAG GCA GGC TCT GCT ACG TCC TGC CTG TGA         1203
Phe Tyr Glu Glu Asp Glu Ala Gly Ser Ala Thr Ser Cys Leu  *
        375                 380                 385
```

AAGAATCTCT TCAGGAAACC AGAGCTTCCC TCATTTACCT TTTCTCCTAC AAAGGGAAGC 1263

AGCCTGGAAG AAACAGTCCA GTACTTGACC CAT 1296

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 386 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Gly Leu Trp Gly Gln Ser Val Pro Thr Ala Ser Ser Ala Arg Ala
 1               5                  10                  15

Gly Arg Tyr Pro Gly Ala Arg Thr Ala Ser Gly Thr Arg Pro Trp Leu
            20                  25                  30

Leu Asp Ser Lys Ile Leu Lys Phe Val Val Phe Ile Val Ala Val Leu
        35                  40                  45

Leu Pro Val Arg Val Asp Ser Ala Thr Ile Pro Arg Gln Asp Glu Val
    50                  55                  60

Pro Gln Gln Thr Val Ala Pro Gln Gln Gln Arg Arg Ser Leu Lys Glu
65                  70                  75                  80

Glu Glu Cys Pro Ala Gly Ser His Arg Ser Glu Tyr Thr Gly Ala Cys
                85                  90                  95

Asn Pro Cys Thr Glu Gly Val Asp Tyr Thr Ile Ala Ser Asn Asn Leu
            100                 105                 110

Pro Ser Cys Leu Leu Cys Thr Val Cys Lys Ser Gly Gln Thr Asn Lys
        115                 120                 125

Ser Ser Cys Thr Thr Thr Arg Asp Thr Val Cys Gln Cys Glu Lys Gly
    130                 135                 140

Ser Phe Gln Asp Lys Asn Ser Pro Glu Met Cys Arg Thr Cys Arg Thr
145                 150                 155                 160

Gly Cys Pro Arg Gly Met Val Lys Val Ser Asn Cys Thr Pro Arg Ser
                165                 170                 175

Asp Ile Lys Cys Lys Asn Glu Ser Ala Ala Ser Ser Thr Gly Lys Thr
            180                 185                 190

Pro Ala Ala Glu Glu Thr Val Thr Thr Ile Leu Gly Met Leu Ala Ser
        195                 200                 205

Pro Tyr His Tyr Leu Ile Ile Ile Val Val Leu Val Ile Ile Leu Ala
    210                 215                 220

Val Val Val Val Gly Phe Ser Cys Arg Lys Lys Phe Ile Ser Tyr Leu
225                 230                 235                 240

Lys Gly Ile Cys Ser Gly Gly Gly Gly Gly Pro Glu Arg Val His Arg
                245                 250                 255
```

```
Val Leu Phe Arg Arg Arg Ser Cys Pro Ser Arg Val Pro Gly Ala Glu
            260                 265                 270

Asp Asn Ala Arg Asn Glu Thr Leu Ser Asn Arg Tyr Leu Gln Pro Thr
        275                 280                 285

Gln Val Ser Glu Gln Glu Ile Gln Gly Gln Glu Leu Ala Glu Leu Thr
    290                 295                 300

Gly Val Thr Val Glu Leu Pro Glu Glu Pro Gln Arg Leu Leu Glu Gln
305                 310                 315                 320

Ala Glu Ala Glu Gly Cys Gln Arg Arg Arg Leu Leu Val Pro Val Asn
                325                 330                 335

Asp Ala Asp Ser Ala Asp Ile Ser Thr Leu Leu Asp Ala Ser Ala Thr
            340                 345                 350

Leu Glu Glu Gly His Ala Lys Glu Thr Ile Gln Asp Gln Leu Val Gly
        355                 360                 365

Ser Glu Lys Leu Phe Tyr Glu Glu Asp Glu Ala Gly Ser Ala Thr Ser
    370                 375                 380

Cys Leu  *
385
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vii) IMMEDIATE SOURCE:
(B) CLONE: FLAG peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Asp Tyr Lys Asp Asp Asp Asp Lys
1               5
```

What is claimed is:

1. An isolated DNA comprising a nucleotide sequence encoding a TRAIL receptor polypeptide (TRAIL-R), wherein said TRAIL-R is selected from the group consisting of:

a) the TRAIL-R polypeptide of Figure 1;

b) the TRAIL-R polypeptide of Figure 2; and

c) a fragment of the polypeptide of (a) or (b), wherein said fragment is capable of binding TRAIL.

2. A DNA of claim 1, wherein said fragment is a soluble TRAIL-R polypeptide.

3. A DNA of claim 2, wherein said soluble TRAIL-R polypeptide comprises the extracellular domain of the TRAIL-R of Figure 1 or 2.

4. A DNA of claim 1, wherein said TRAIL-R lacks a transmembrane region.

5. A DNA of claim 1, wherein said TRAIL-R polypeptide comprises the amino acid sequence of residues x to y of Figure 1 or Figure 2, wherein x represents an integer from 1 to 98, and y represents an integer from 181 to 386.

6. A DNA of claim 5, wherein x is an integer selected from the group consisting of 1 and 56, and y is an integer selected from the group consisting of 181, 208, 211, and 386.

7. An isolated DNA encoding a TRAIL-R polypeptide, wherein said polypeptide comprises an amino acid sequence that is at least 90% identical to an amino acid sequence selected from the group consisting of:

a) residues 1 to 386 of Figure 1;

b) residues 1 to 386 of Figure 2;

c) residues 56 to 386 of Figure 1;

d) residues 56 to 386 of Figure 2;

e) residues 1 to 211 of Figure 1;

f) residues 1 to 211 of Figure 2; and

g) residues 56 to 211 of Figure 1.

b) residues 1 to 386 of Figure 2;

c) residues 56 to 386 of Figure 1;

d) residues 56 to 386 of Figure 2;

e) residues 1 to 211 of Figure 1;

f) residues 1 to 211 of Figure 2; and

g) residues 56 to 211 of Figure 1.

9. An isolated DNA comprising at least 60 contiguous nucleotides of the nucleotide sequence of Figure 1 or Figure 2.

10. An expression vector comprising a DNA according to claim 1, 2, 3, 5, 7, or 8.

11. A process for preparing a TRAIL-R polypeptide, comprising culturing a host cell containing a vector according to claim 10 under conditions promoting expression of TRAIL-R, and recovering the TRAIL-R polypeptide.

12. A purified TRAIL-R polypeptide selected from the group consisting of:

a) the TRAIL-R polypeptide of Figure 1 in mature form;

b) the TRAIL-R polypeptide of Figure 2 in mature form; and

c) a fragment of the polypeptide of (a) or (b), wherein said fragment is capable of binding TRAIL.

13. A TRAIL-R of claim 12, wherein said fragment is a soluble TRAIL-R polypeptide.

14. A TRAIL-R of claim 13, wherein said soluble TRAIL-R polypeptide comprises the extracellular domain of the TRAIL-R of Figure 1 or 2.

15. A TRAIL-R of claim 12, wherein said TRAIL-R lacks a transmembrane region.

16. A TRAIL-R of claim 12, wherein said TRAIL-R polypeptide comprises the amino acid sequence of residues x to y of Figure 1 or Figure 2, wherein x represents an integer from 1 to 98, and y represents an integer from 181 to 386.

17. A TRAIL-R of claim 16, wherein x is an integer selected from the group consisting of 1 and 56, and y is an integer selected from the group consisting of 181, 208, 211, and 386.

18. A purified TRAIL-R polypeptide comprising an amino acid sequence that is at least 90% identical to an amino acid sequence selected from the group consisting of:

a) residues 56 to 386 of Figure 1;

b) residues 56 to 386 of Figure 2; and

c) residues 56 to 211 of Figure 1.

19. A TRAIL-R of claim 18, wherein said polypeptide comprises an amino acid sequence selected from the group consisting of:

a) residues 56 to 386 of Figure 1;

b) residues 56 to 386 of Figure 2; and

c) residues 56 to 211 of Figure 1.

20. An oligomer comprising at least two TRAIL-R polypeptides of claim 12, 13, 18, or 19.

21. An oligomer of claim 20, wherein said oligomer comprises from two to four soluble TRAIL-R polypeptides.

22. A fusion protein comprising a TRAIL-R polypeptide and an antibody-derived Fc polypeptide, wherein said TRAIL-R polypeptide is a soluble fragment of the TRAIL-R protein of Figure 1 or Figure 2, wherein said fragment is capable of binding TRAIL.

23. A dimer comprising two fusion proteins of claim 22.

24. A composition comprising a polypeptide of claim 12, and a physiologically acceptable diluent, excipient, or carrier.

25. A composition comprising an oligomer of claim 20, and a physiologically acceptable diluent, excipient, or carrier.

26. An antibody that is directed against a TRAIL-R polypeptide of claim 12, or an antigen-binding fragment of said antibody.

27. An antibody of claim 26, wherein said antibody is a monoclonal antibody.

FIGURE 1A

```
TGATTGATTTTTGGCGCTTTCGATCCACCCTCCTCCCTTCTCATGGGACTTTGGGGACAA
                                          M  G  L  W  G  Q

AGCGTCCCGACCGCCTCGAGCGCTCGAGCAGGGCGCTATCCAGGAGCCAGGACAGCGTCG
S  V  P  T  A  S  S  A  R  A  G  R  Y  P  G  A  R  T  A  S

GGAACCAGACCATGGCTCCTGGACCCCAAGATCCTTAAGTTCGTCGTCTTCATCGTCGCG
G  T  R  P  W  L  L  D  P  K  I  L  K  F  V  V  F  I  V  A

GTTCTGCTGCCGGTCCGGGTTGACTCTGCCACCATCCCCCGGCAGGACGAAGTTCCCCAG
V  L  L  P  V  R  V  D  S  A  T  I  P  R  Q  D  E  V  P  Q

CAGACAGTGGCCCCACAGCAACAGAGGCGCAGCCTCAAGGAGGAGGAGTGTCCAGCAGGA
Q  T  V  A  P  Q  Q  Q  R  R  S  L  K  E  E  E  C  P  A  G

TCTCATAGATCAGAATATACTGGAGCCTGTAACCCGTGCACAGAGGGTGTGGATTACACC
S  H  R  S  E  Y  T  G  A  C  N  P  C  T  E  G  V  D  Y  T

ATTGCTTCCAACAATTTGCCTTCTTGCCTGCTATGTACAGTTTGTAAATCAGGTCAAACA
I  A  S  N  N  L  P  S  C  L  L  C  T  V  C  K  S  G  Q  T

AATAAAAGTTCCTGTACCACGACCAGAGACACCGTGTGTCAGTGTGAAAAAGGAAGCTTC
N  K  S  S  C  T  T  T  R  D  T  V  C  Q  C  E  K  G  S  F

CAGGATAAAAACTCCCCTGAGATGTGCCGGACGTGTAGAACAGGGTGTCCCAGAGGGATG
Q  D  K  N  S  P  E  M  C  R  T  C  R  T  G  C  P  R  G  M

GTCAAGGTCAGTAATTGTACGCCCCGGAGTGACATCAAGTGCAAAAATGAATCAGCTGCC
V  K  V  S  N  C  T  P  R  S  D  I  K  C  K  N  E  S  A  A

AGTTCCACTGGGAAAACCCCAGCAGCGGAGGAGACAGTGACCACCATCCTGGGGATGCTT
S  S  T  G  K  T  P  A  A  E  E  T  V  T  T  I  L  G  M  L

GCCTCTCCCTATCACTACCTTATCATCATAGTGGTTTTAGTCATCATTTTAGCTGTGGTT
A  S  P  Y  H  Y  L  I  I  I  V  V  L  V  I  I  L  A  V  V

GTGGTTGGCTTTTCATGTCGGAAGAAATTCATTTCTTACCTCAAAGGCATCTGCTCAGGT
V  V  G  F  S  C  R  K  K  F  I  S  Y  L  K  G  I  C  S  G
```

FIGURE 1B

```
GGTGGAGGAGGTCCCGAACGTGTGCACAGAGTCCTTTTCCGGCGGCGTTCATGTCCTTCA
G  G  G  G  P  E  R  V  H  R  V  L  F  R  R  R  S  C  P  S

CGAGTTCCTGGGGCGGAGGACAATGCCCGCAACGAGACCCTGAGTAACAGATACTTGCAG
R  V  P  G  A  E  D  N  A  R  N  E  T  L  S  N  R  Y  L  Q

CCCACCCAGGTCTCTGAGCAGGAAATCCAAGGTCAGGAGCTGGCAGAGCTAACAGGTGTG
P  T  Q  V  S  E  Q  E  I  Q  G  Q  E  L  A  E  L  T  G  V

ACTGTAGAGTCGCCAGAGGAGCCACAGCGTCTGCTGGAACAGGCAGAAGCTGAAGGGTGT
T  V  E  S  P  E  E  P  Q  R  L  L  E  Q  A  E  A  E  G  C

CAGAGGAGGAGGCTGCTGGTTCCAGTGAATGACGCTGACTCCGCTGACATCAGCACCTTG
Q  R  R  R  L  L  V  P  V  N  D  A  D  S  A  D  I  S  T  L

CTGGATGCCTCGGCAACACTGGAAGAAGGACATGCAAAGGAAACAATTCAGGACCAACTG
L  D  A  S  A  T  L  E  E  G  H  A  K  E  T  I  Q  D  Q  L

GTGGGCTCCGAAAAGCTCTTTTATGAAGAAGATGAGGCAGGCTCTGCTACGTCCTGCCTG
V  G  S  E  K  L  F  Y  E  E  D  E  A  G  S  A  T  S  C  L

TGAAAGAATCTCTTCAGGAAACCAGAGCTTCCCTCATTTACCTTTTCTCCTACAAAGGGA
*

AGCAGCCTGGAAGAAACAGTCCAGTACTTGACCCATGCCCCAACAAACTCTACTATCCAA

TATGGGGCAGCTTACCAATGGTCCTAGAACTTTGTTAACGCACTTGGAGTAATTTTTATG

AAATACTGCGTGTGATAAGCAAACGGGAGAAATTTATATCAGATTCTTGGCTGCATAGTT

ATACGATTGTGTATTAAGGGTCGTTTTAGGCCACATGCGGTGGCTCATGCCTGTAATCCC

AGCACTTTGATAGGCTGAGGCAGGTGGATTGCTTGAGCTCGGGAGTTTGAGA
```

FIGURE 2A

```
                                                                  .
TGATTGATTTTTGGCGCTTTCGATCCACCCTCCTCCCTTCTCATGGGACTTTGGGGACAA
                                                  M  G  L  W  G  Q

                                                                  .
AGCGTCCCGACCGCCTCGAGCGCTCGAGCAGGGCGCTATCCAGGAGCCAGGACAGCGTCG
S  V  P  T  A  S  S  A  R  A  G  R  Y  P  G  A  R  T  A  S

                                                                  .
GGAACCAGACCATGGCTCCTGGACTCCAAGATCCTTAAGTTCGTCGTCTTCATCGTCGCG
G  T  R  P  W  L  L  D  S  K  I  L  K  F  V  V  F  I  V  A

                                                                  .
GTTCTGCTGCCGGTCCGGGTTGACTCTGCCACCATCCCCCGGCAGGACGAAGTTCCCCAG
V  L  L  P  V  R  V  D  S  A  T  I  P  R  Q  D  E  V  P  Q

                                                                  .
CAGACAGTGGCCCCACAGCAACAGAGGCGCAGCCTCAAGGAGGAGGAGTGTCCAGCAGGA
Q  T  V  A  P  Q  Q  Q  R  R  S  L  K  E  E  E  C  P  A  G

                                                                  .
TCTCATAGATCAGAATATACTGGAGCCTGTAACCCGTGCACAGAGGGTGTGGATTACACC
S  H  R  S  E  Y  T  G  A  C  N  P  C  T  E  G  V  D  Y  T

                                                                  .
ATTGCTTCCAACAATTTGCCTTCTTGCCTGCTATGTACAGTTTGTAAATCAGGTCAAACA
I  A  S  N  N  L  P  S  C  L  L  C  T  V  C  K  S  G  Q  T

                                                                  .
AATAAAAGTTCCTGTACCACGACCAGAGACACCGTGTGTCAGTGTGAAAAAGGAAGCTTC
N  K  S  S  C  T  T  T  R  D  T  V  C  Q  C  E  K  G  S  F

                                                                  .
CAGGATAAAAACTCCCCTGAGATGTGCCGGACGTGTAGAACAGGGTGTCCCAGAGGGATG
Q  D  K  N  S  P  E  M  C  R  T  C  R  T  G  C  P  R  G  M

                                                                  .
GTCAAGGTCAGTAATTGTACGCCCCGGAGTGACATCAAGTGCAAAAATGAATCAGCTGCC
V  K  V  S  N  C  T  P  R  S  D  I  K  C  K  N  E  S  A  A

                                                                  .
AGTTCCACTGGGAAAACCCCAGCAGCGGAGGAGACAGTGACCACCATCCTGGGGATGCTT
S  S  T  G  K  T  P  A  A  E  E  T  V  T  T  I  L  G  M  L

                                                                  .
GCCTCTCCCTATCACTACCTTATCATCATAGTGGTTTTAGTCATCATTTTAGCTGTGGTT
A  S  P  Y  H  Y  L  I  I  I  V  V  L  V  I  I  L  A  V  V

                                                                  .
GTGGTTGGCTTTTCATGTCGGAAGAAATTCATTTCTTACCTCAAAGGCATCTGCTCAGGT
V  V  G  F  S  C  R  K  K  F  I  S  Y  L  K  G  I  C  S  G
```

FIGURE 2B

```
                .         .         .         .         .         .
GGTGGAGGAGGTCCCGAACGTGTGCACAGAGTCCTTTTCCGGCGGCGTTCATGTCCTTCA
G  G  G  G  P  E  R  V  H  R  V  L  F  R  R  R  S  C  P  S

                .         .         .         .         .         .
CGAGTTCCTGGGGCGGAGGACAATGCCCGCAACGAGACCCTGAGTAACAGATACTTGCAG
R  V  P  G  A  E  D  N  A  R  N  E  T  L  S  N  R  Y  L  Q

                .         .         .         .         .         .
CCCACCCAGGTCTCTGAGCAGGAAATCCAAGGTCAGGAGCTGGCAGAGCTAACAGGTGTG
P  T  Q  V  S  E  Q  E  I  Q  G  Q  E  L  A  E  L  T  G  V

                .         .         .         .         .         .
ACTGTAGAGTTGCCAGAGGAGCCACAGCGTCTGCTGGAACAGGCAGAAGCTGAAGGGTGT
T  V  E  L  P  E  E  P  Q  R  L  L  E  Q  A  E  A  E  G  C

                .         .         .         .         .         .
CAGAGGAGGAGGCTGCTGGTTCCAGTGAATGACGCTGACTCCGCTGACATCAGCACCTTG
Q  R  R  R  L  L  V  P  V  N  D  A  D  S  A  D  I  S  T  L

                .         .         .         .         .         .
CTGGATGCCTCGGCAACACTGGAAGAAGGACATGCAAAGGAAACAATTCAGGACCAACTG
L  D  A  S  A  T  L  E  E  G  H  A  K  E  T  I  Q  D  Q  L

                .         .         .         .         .         .
GTGGGCTCCGAAAAGCTCTTTTATGAAGAAGATGAGGCAGGCTCTGCTACGTCCTGCCTG
V  G  S  E  K  L  F  Y  E  E  D  E  A  G  S  A  T  S  C  L

                .         .         .         .         .         .
TGAAAGAATCTCTTCAGGAAACCAGAGCTTCCCTCATTTACCTTTTCTCCTACAAAGGGA
*

                .         .         .
AGCAGCCTGGAAGAAACAGTCCAGTACTTGACCCAT
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14410

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/12, 15/62; C07K 14/715, 16/28, 16/46; A61K 38/17
US CL :536/23.5; 435/69.1, 69.7, 320.1; 530/350, 388.22; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/69.1, 69.7, 320.1; 530/350, 388.22; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Keyword databases: Medline, USPTO-APS
Search terms: TRAIL, Apo-2, Apo-2L; receptor
Sequence databases: GenBank/EMBL/DDBJ, GeneSeq. Swissprot, PIR

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y - A	PAN, G. et al. The receptor for the cytotoxic ligand TRAIL. Science. 04 April 1997, Vol. 276, pages 111-113, especially Figure 1a.	26, 27 --- 1-25
X, P --- Y, P --- A,P	MARSTERS, S.A. et al. A novel receptor for Apo2L/TRAIL contains a truncated death domain. Current Biology. December 1997, Vol. 7, No. 12, pages 1003-1006, especially Figure 1(a).	1, 5-11 --- 2-4, 12-19, 24, 26, 27
A,P	GOLSTEIN, P. Cell death: TRAIL and its receptors. Current Biology. December 1997, Vol. 7, No. 12, pages R750-R753.	1-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 29 OCTOBER 1998	Date of mailing of the international search report 04 NOV 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID L. FITZGERALD Telephone No. (703) 308-0196

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